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The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 43 JANUARY, 1954 NO. 1

The Official Publication of the Central Society for Clinical Research

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The Journal of Laboratory and Clinical Medicine

VOL. 43 JANUARY, 1954 No. 1

LETTER FROM THE EDITOR

AT THE beginning of another year, the Editors and members of the Editorial board again wish to take this opportunity to express their appreciation and thanks for the help they have received from their colleagues and many others, both members and nonmembers, of the Central Society for Clinical Research in providing critical appraisal of manuscripts submitted to the Journal for publication. The individuals whose names are listed below have given freely of their time and have rendered valuable editorial assistance during the past year.

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On July 1, 1953, Dr. Matthew Block left the University of Chicago to accept a position on the faculty of medicine at the University of Colorado School of Medicine. The Editor wishes to take this opportunity to thank him for the excellent cooperation and valuable service he gave to the Journal and Society during his time as Associate Editor. The Editor is happy to announce that Dr. Robert H. Ebert, Associate Professor of Medicine, accepted the appointment as Associate Editor to replace Dr. Block.

As of Dec. 30, 1953, Drs. William B. Bean, Kenneth M. Brinkhous, and Carl V. Moore, having served two three-year terms, retired from the Board. On behalf of the Society and the Mosby Company, the Editors take this opportunity to thank them for their valuable services and to welcome Drs. Frank H. Bethell, Elmer L. DeGowin, and Oliver H. Lowry, who will replace them on the Board for a three-year term beginning Jan 1, 1953. Since the JOURNAL OF LABORATORY AND CLINICAL MEDICINE became the official publication of the Central Society for Clinical Research, Dr. Moore has played a principal role—first as Editor and later as an Editorial Board member—in bringing it to its present high level in the field of scientific journals. Dr. Thomas H. Hunter has accepted a second three-year term appointment.

The readers of the Journal as well as those submitting papers to it for possible publication are directed to the thought-provoking leading article by Dr. George E. Burch entitled "Of Publishing Scientific Papers." Serious consideration of the statements made by Dr. Burch can only serve to improve the scientific accuracy of papers and thereby make the work of the Editor and the Board much easier.

CLAYTON G. LOOSLI, M.D.
Editor

OF PUBLISHING SCIENTIFIC PAPERS

G. E. Burch, M.D.
New Orleans, La.

TO CRYSTALLIZE successfully one's researches into a concise, lucid, well-organized, and well-illustrated paper is an achievement limited to few investigators. Such a publication results in intellectual gratification to both author and reader. To read and study well-written papers is a joy of which the reader is always appreciative.

A good scientific paper, first, contains new data well oriented among those already in the literature and, second, accurately evaluates the new contributions. It indicates the purpose of the investigation, describes the experiments, and presents precisely the origin of the ideas. The results are summarized clearly but without modification, selection, or elimination of data for the purpose of reaching preconceived, prejudiced, or apparently acceptable conclusions. The arguments by which the interpretations were logically and clearly made are included in the paper, to permit the readers to judge all aspects. If the investigation has been properly conducted and if the methods are adequately described, the experiments may be repeated by others, and, within the limits of existing knowledge, essentially the same interpretations can be made. Furthermore, the disciplinary effect of taking periodic inventory in research by writing for publication has its scientific rewards to the investigator and alone is sufficient to make obligatory the writing, but not necessarily the publishing of scientific papers in research.

It should be noted that whereas medical science has progressed and the medical literature has expanded considerably, methods of publication have not enjoyed commensurate development, improvement, and attention. A critical review of some aspects of present-day practices in medical writing may be helpful, even though adequate solutions for current problems, errors, and deficiencies are not offered. It is hoped, nevertheless, that these reflections may provoke critical and corrective meditation among those concerned directly and indirectly with medical writing.

The *investigator-author* is *primarily responsible* for the production of the paper. The long succession of others concerned with its production, including benefactor, institution, editor, and publisher, should insist on a superior product and should aid the author in every way to accomplish this end. Like his researches, the investigator's writing should be initiated spontaneously to disclose to the scientific world scientifically accurate and theoretically sound ideas which constitute a *real* contribution. The investigator should be honestly convinced, from a thorough knowledge of the literature and his own researches, that at least these minimal requirements for his paper exist. Pub-

Presidential address, Twenty-sixth Annual Meeting of the Central Society for Clinical Research, Chicago, Ill., Oct. 30, 1953.

From the Department of Medicine, Tulane University School of Medicine, and Charity Hospital of Louisiana at New Orleans.

lications prompted by motives of self-aggrandizement or other selfish considerations are rarely accurate or original. Repetitive publication of the same data or ideas for different journals, foreign or national, reflects scientific sterility and constitutes exploitation of what is considered an ethical medium for propagandizing one's self (Fig. 1). Self-plagiarism signifies lack of scientific

Fig. 1.—The Self-plagiarist.

Fig. 2.—The Self-aggrandizer.

objectivity and modesty. For enhancement of significance and to become established, scientific observations should be corroborated by others, not by one's self. Priority accompanies the earliest publication and is not acquired or more firmly established by repeated publications of essentially the same data.

Writing in science to be fashionable, to qualify for membership in a society, or for academic and financial advancement or self-glorification is unpardonable (Fig. 2). These personal gains constitute the inevitable reward of commendable work, not the goal. True scientific writing cloaks a humble, modest character. Papers solicited, especially under pressure, by friends, editors of journals, or medical societies are likewise rarely of maximal quality.

Regardless of origin, all previously published reports must be reviewed before the investigator may consider his data original (Fig. 3). Failure to establish the accuracy and originality of one's own data, with proper perspective within the whole scientific framework, reflects lack of scientific maturity. Only the absolute truth can be tolerated in science; no compromise is permissible. There is no substitute for observation by scientific methods as the final test in establishing a scientific truth. Furthermore, the application of elaborate graphs, charts, mathematical and statistical tools, or eloquent language to inaccurate data cannot render them valid. Fortunately, the informed, critical, and capable reader is rarely misled. Publications which are unreliable

Fig. 3.—Tubular Vision.

are to be condemned. Similarly, the investigator who is prone to claim or to imply cures for disease states without providing conclusive evidence should be censured. The recent medical literature on chronic disease is replete with such examples. The energies of many men expended in disproving false claims could be devoted to more positive endeavors.

The author should be permitted to present his observations and ideas, which constitute the nucleus of any scientific paper, in his own style without major alteration by publication policies or editorial disciplines. The scientific character of a man is identifiable in his writing and often assists in evaluation of his work by others. To be sure, a good literary style is enviable, but experimental and scientific inadequacies can be deceivingly cloaked in literary eloquence. For the true investigator, research is an addiction from which his mind cannot peacefully escape. He is forever objectively observing and analyzing events about him, and these experiences should be recorded in his own best fashion.

Scientific ability should be judged by the *quality,* not the *quantity,* of the publications (Fig. 4). It is likewise unsound for the inactive investigator to rationalize his sterility by the proclamation that extensive writing cannot be of high quality. Scientific writing, like scientific data, must be judged on its own merits through careful study. To a fortunate few, research comes with the apparent ease of a craft to the professional craftsman. Shakespeare's writings, prolific though they were, are not considered inferior, nor is the work of Gauss considered poor because his interests were so extensive.

Fig. 4.—The Obsessive Writer.

Fig. 5.—The Scanner.

Papers should be constructed to facilitate reading. Only clear, definite statements should be presented. Remember that the writer has all his data, whereas the reader has only those presented to him, and in a fashion chosen by the author. All sentences, therefore, should lucidly convey to the reader the precise thoughts and images in the mind of the writer. All pertinent data should be included, especially the method of study, which, with present-day emphasis on brevity, is unfortunately often not described in detail. By reviewing the original data, the experienced investigator can properly evaluate a presentation. Furthermore, the experimental details should be made available to those who wish to repeat the experiments, a privilege frequently denied colleagues today. Accuracy and thoroughness can never be sacrificed in writing.

The *reader* of scientific papers, as the audience of the writer, should discharge with diligence the important responsibility associated with the privilege of reading scientific papers. He should tolerate neither inaccurate and unfounded data nor careless interpretations and discussions. He should study the papers analytically, critically, and with the devotion to scientific thought

that he would exercise were he himself producing an article from the data available in the paper. For proper evaluation, papers in science must be examined like a document, carefully, deliberately, objectively, and without prejudice or emotion. A scientific paper must be studied, not read. Just as there are few men who can do scientific work well and write properly, there are few who have the attributes for proper study of scientific papers. Only after deliberate examination is one capable of satisfactorily evaluating the paper and thus entitled to the privilege of criticizing it (Fig. 5). Then, he should express his opinions, whether laudatory or deprecatory. Readers are frequently eager to comment strongly about the merits of data and papers which they have not examined carefully (Fig. 6). Criticism should be the privilege of those who have more than cursory impressions.

Fig. 6.—The Critic.

Fig. 7.—The Summary Addict.

Clinical papers should also be read carefully, especially those concerned with new ideas in therapy. Such analysis would preclude many erroneous contentions from progressing beyond the publication stage. The practice of reading the summary alone, often written on the basis of a few cases with apologies but with implications of finality, frequently results in general use of the ideas by the reader rather than a demand for more conclusive data from the same author to establish the truth (Fig. 7). The reader should employ his imagination to the utmost when studying a paper but that must be recognized as his own contribution to the "advancement of the scientific literature."

One must be fully informed on a subject to be capable of adequate criticism. Mathematical expressions and other such auxiliary tools, including the significance of the assumptions in the interpretations, must be understood by the reader. However, unless sufficient details of method and original data have been presented by the author, the reader is obliged to accept or discard results, interpretations, and conclusions on the basis of his confidence in the investigator or *apparent* reasonableness of the presentation. Without

adequate data the reader is furthermore deprived of the most interesting aspect of study of the paper, i.e., critical reading, which, like critical discussion, leads to scientific advancement. To read critically is to conduct a critical conference with the author, who has committed himself "in writing."

The *benefactor, university,* and *other similar sponsors of research* also have an important responsibility in scientific writing, and that is to provide guidance and assistance in producing only publications of the highest quality. This

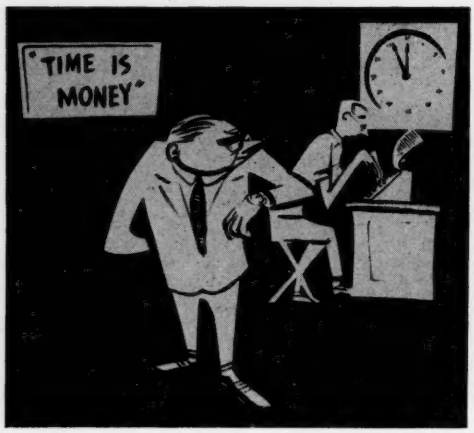

Fig. 8.—The Prodder.

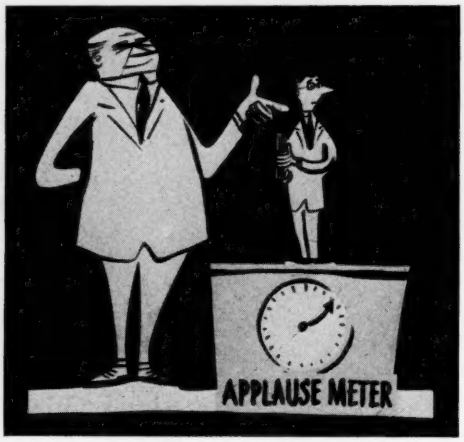

Fig. 9.—The Evaluator.

can best be fostered by granting unlimited freedom and support to the capable investigator in his research and writing, regardless of the immediate practical aspects of the studies. He should therefore never be prodded for premature publications (Fig. 8). Institutions should refrain from evaluating the investigator and the quality of his publications on the basis of the uninformed layman's response or of general acclaim or condemnation (Fig. 9). That a scientific truth cannot be established by public opinion is evidenced by the experiences of Galileo when he declared that the earth was round.

The journal *editor* (Fig. 10) and all his assistants also bear an important responsibility in medical publications. Their qualifications should include scientific knowledge, irreproachable scientific interest, objectivity, industry, critical judgment, and ability to write scientifically themselves. They should be scientifically humble, unprejudiced, intellectually honest, and concerned with aiding, not retarding, the advancement of science or the investigator. The editor and his assistants should conduct themselves in a fashion to indicate interest in assisting the investigator and not in impeding him. Policies should never be established by edict. Nor should the editor criticize methods, data, analytic procedures or interpretations after only cursory perusal of a paper to which the investigator has devoted hours of thought and laboratory

research (Fig. 11). Only the investigator can write accurately about his own scientific experiences. Above all, the slightest modification, regardless of its apparent lack of significance, should be approved by the author.

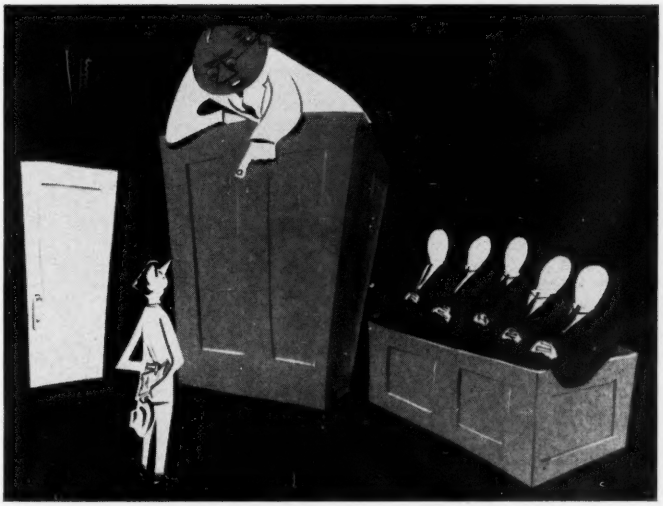

Fig. 10.—The Editor and his Board.

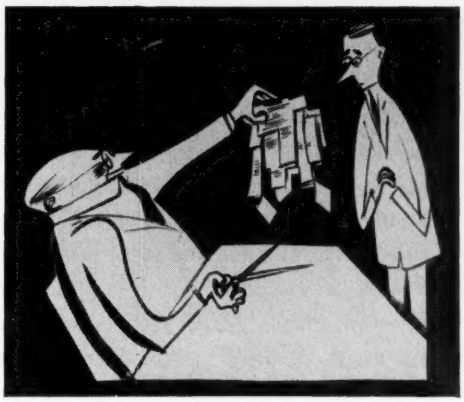

Fig. 11.—The Censor.

The editor should exercise his convictions and opinions not dogmatically but humbly, conservatively, and in an unbiased, courteous, though positive, manner. When the merit of a paper remains dubious in the minds of the editor and capable consultants, he should so inform the investigator, who should then be given the opportunity of publishing the paper, if he wishes, allowing the readers and the test of time ultimately to establish the merits of the data and interpretations.

A capable editor, on the other hand, is quick to recognize the scientific charlatan. He is sufficiently perceptive to detect readily papers inadequately oriented in the existing stage of knowledge and to recognize self-plagiarism, borrowed material, poorly substantiated conclusions, and disorganized presentations.

Fig. 12.—The Man of Distinction.

Fig. 13.—The Board-o-philiac.

Ideally, any member of the editorial board should be qualified to serve as editor. Not "retired" scientists, but active vigorous investigators should hold these appointments (Fig. 12). They should not be like inhibitors but catalysts. It is they and the editor, and not the editorial committee, who should be primarily responsible for adoption and execution of all important policies of the journals. A good editorial board should manage and direct administrative activities of the journal. The greater the division of responsibility and authority, the poorer the service to science, for responsibility without authority can only result in failure.

The individual who appreciates fully the responsibilities of an editorial board will accept appointment to only one or two journals, to which he can devote the necessary time to perform a good service (Fig. 13). Those who are too occupied with other endeavors far removed from active participation in research and critical scientific thought and who hold appointments on editorial boards in name only act as "window dressers." Appointment to an editorial board for purposes of self-glorification rather than for superior scientific and editorial abilities is an affront, not an honor. The dictatorial editor whose editorial board functions as "mannequins" and who makes most of the important decisions himself is extremely limited in his accomplishments (Fig. 14). In general, then, the usefulness of a member of an editorial board is in-

versely and exponentially related to the number of journals he serves. The "influential" or "important" people who are appointed to editorial boards for economic or medical political, rather than scientific, reasons more honestly belong in the category of "Editorial Sponsors"—analogous to civic leaders who sponsor municipal philharmonic societies. Such a body would, however, seem to have a limited place in the field of true science. It should always be remembered that the investigator and his scientific data are of paramount, not secondary, importance to editors, boards, committees, publishing companies, or the scientific society.

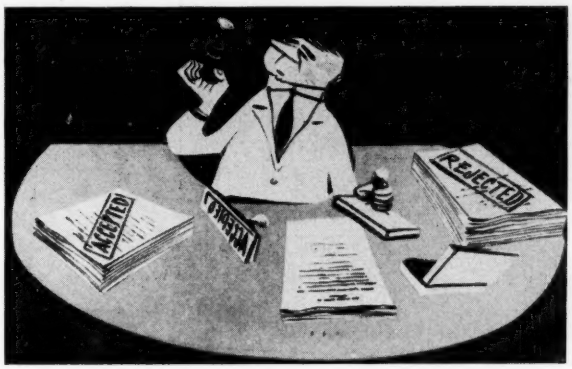

Fig. 14.—The Dictator.

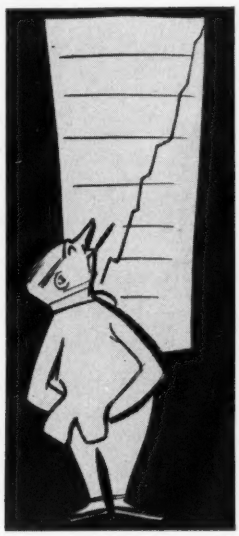

Fig. 15.—The Stockholder.

The rotation of editors and members of the editorial boards at three- to five-year intervals should be mandatory. The membership should comprise representatives of the major disciplines covered by the papers submitted, outside scientists being consulted for assistance on highly specialized subjects. Membership on an active, properly functioning board of diligent scientists should constitute an honor and provide intellectual pleasure.

The *publishing company* or publisher has a responsibility to science and the scientists which exceeds that to the stockholders. Surely, the publisher is entitled to a profit, but not at the expense of science or a compromise in quality or structure of the editorial personnel and publications (Fig. 15). Journals owned by strictly scientific institutions have the definite advantage of not being completely governed by economic pressure demanding a financial profit. Advertising in the journal, if permitted, should conform to accepted

ethics, an enforcement often initiated and maintained by good publishing companies rather than the board of editors or medical organizations themselves (Fig. 16).

Publishers should constantly work toward improvement in illustrations, typesetting, paper, spacing, and other aspects of format to keep abreast of other advances in science and research which assist the investigator in presenting his material more effectively. In such endeavors as the publication of monographs, in which the publisher and author work alone, the integrity of the publisher assumes paramount importance.

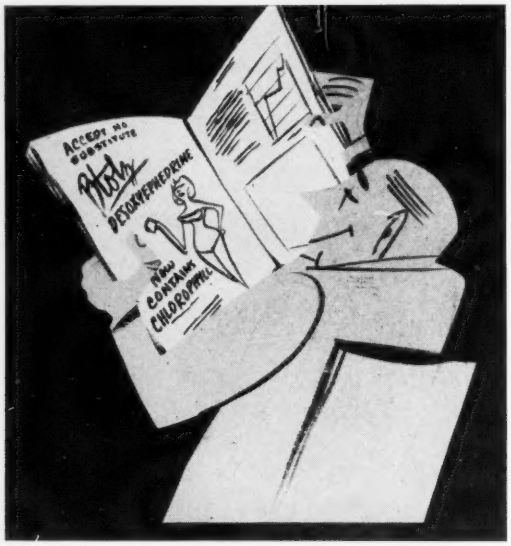

Fig. 16.—The Advertiser.

Considerable improvement is needed in scientific publications, particularly in state medical journals. Today, with greater emphasis on political and economic problems, they often contain clinical material which has been rejected by the better national journals. These local journals, because of the need to fill their progressively thinning issues, tend to encourage publication of poor individual case reports and other inferior material. Interestingly enough, mainly "successful" clinical procedures enjoy publication, and seldom does a retraction of previously published inaccuracies appear. Perhaps the state journal which must go to press on schedule with a full issue of clinical papers, regardless of quality, should be supplanted by a nonscientific bulletin containing political, commercial, and administrative activities of the society, and regional rather than state journals should present the limited scientific material of adequate quality.

Finally, science and the scientist should provide the curious laity with accurate reporting of scientific activities in medicine, both clinical and experi-

mental. Newspaper and magazine reporting today is inadequate because of many factors. Sometimes the scientist or physician may permit the reporter, because of local pride, to imply that his activities are original, when actually many others may have been engaged in similar programs long before. The hypomanic pseudoscientist, rather than the humble and conservative scientist directly responsible for the original work, tends to provide the material for lay consumption; this can only lead to inaccurate and sensational reporting aimed at the reader's emotions and large sales rather than truthful reporting designed for education of the reader (Fig. 17). Provisions should be available for the originator of ideas or discoverer himself to furnish such material.

Fig. 17.—The Sensationalist.

Opinions, impressions, ill-conceived ideas, and fantasies about scientific and applied medicine should be clearly differentiated from scientific fact and established truth (Fig. 18). Competent reporting by the large newspapers or syndicates would require a staff of scientists who would not be misled by the newsworthy names in medicine any more than would a good editorial board. Many people who "report" periodically through the newspapers and magazines of "accomplishments" in medical science cannot publish the same "accomplishments" in well-established critical scientific journals. There is a need for lucid, accurate, and objective scientific reporting in lay publications.

Only a brief inventory of the problems of scientific medical writing has been presented with few or no solutions. That the present situation is defective and that the problems deserve careful study and correction, all will agree. Improvement cannot come as a result of controls, but only from demanding the highest integrity and performance among all concerned with writing of scientific papers. The fundamental responsibility for the character of scien-

tific papers resides with the investigator-author. Finally, the practice of requiring papers for qualification for membership in certain societies, to fill journal issues, or to satisfy a demand by certain readers is to be vigorously and actively discouraged. Because research is fashionable today and a lengthy personal bibliography is considered an index of scientific ability, many feel compelled to engage in these endeavors. It is the duty of all to evaluate publications critically and rigidly and to denounce inaccuracies and inferior quality in research and writing.

The Daily Blast
'HOT' PACE OF WOMAN'S LIFE BLAMED FOR CRIPPLING DISEASES
Science Discovers That When It's Hot You Will Perspire
Man Born To Crawl, Says Doctor
Convicts Test New Vaccine For Rabies
Tropical Disease Situation Called Threat to Florida
Why women faint at fittings has one standing explenation

Fig. 18.—The Miracle Monger.

I leave it to you to decide where these criticisms apply, if at all. Each man in science must judge himself as dispassionately as he should judge his data and their implications. It is hoped that these opinions will provoke objective discussion and self-criticism and thus effect improvement.

SUMMARY

From a study today of scientific papers in medicine, one is forced to question the validity of Bacon's statement as applied to all men, that "Writing maketh an exact man."

CLINICAL AND EXPERIMENTAL

ERYTHROMYCIN THERAPY OF RESPIRATORY INFECTIONS.

I. Controlled Studies on the Comparative Efficacy of Erythromycin and Penicillin in Scarlet Fever

Lieutenant Thomas H. Haight, Medical Corps, United States Naval Reserve

THE discovery of erythromycin by McGuire and his associates[1] has prompted considerable investigation of its antibacterial and pharmacologic properties. The antibiotic has been shown to have marked inhibitory effect against Gram-positive organisms in particular, and this bactericidal activity has been associated with definite ameliorating effects on the course of clinical disease as well.[2-6] These early reports on the use of erythromycin in the treatment of diseases produced by susceptible organisms have shown quite clearly that the agent has a definite place in the ever-widening array of useful and available antibiotics. These preliminary clinical trials have, in general, been confined to the study of selected cases and, usually, only small numbers of any one particular disease entity.

Because of the marked similarity in the antibacterial spectra and mode of action of penicillin and erythromycin[7-9] and the apparently similar results thus far reported in the treatment of certain susceptible diseases, it seemed indicated to obtain valid data on the comparative efficacy of the two agents in the therapy of such conditions. Opportunities were available for such controlled studies at two U. S. Naval Training Centers during the winter of 1952-1953. The present report is concerned with the results of the observations made at one of these.*

BACKGROUND

Epidemics of streptococcal infections have occurred frequently at the particular Naval Training Center, necessitating the use of penicillin prophylaxis on two occasions.[10, 11] It became evident in the late fall of 1952 that the

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*The erythromycin (Ilotycin, Lilly) used in this study was generously supplied by Dr. J. W. Smith of the Eli Lilly Research Laboratories, Indianapolis, Ind.

admission rates for acute respiratory diseases were increasing in the recruit population, and it was noted that a major factor in the increase was due to the prevalence of scarlet fever, exudative tonsillitis with throat cultures positive for hemolytic streptococci, cases diagnosed as tonsillitis-pharyngitis, and a relatively high percentage of recruits carrying streptococci in the nose and throat.

The general incidence of presumptive streptococcal infections and of scarlet fever continued high, and it was therefore felt that sufficient case material would be available to institute a carefully controlled evaluation of the comparative efficacy of erythromycin and procaine penicillin in the treatment of scarlet fever. Actually, the opportunity was unique, inasmuch as it is rarely possible to study a relatively "pure" disease. In this instance, the fact that all the cases of scarlet fever could be highly selected and were almost wholly caused by a group A, type 19 beta hemolytic streptococcus helped to insure that any variation in results would not likely be due to variations in the disease pattern produced by different strains of streptococci.

No attempt has been made to quantitate the results in terms of the dosages used of either antibiotic. The purpose of the study was to determine if erythromycin given in adequate amounts by mouth could duplicate the results which had been previously obtained with penicillin by many workers.[12-18]

PATIENTS, MATERIALS, AND METHODS

Selection of Cases.—All of the patients included in this study were treated on the isolation wards of the U. S. Naval Hospital, Bainbridge, Maryland, during January and February, 1953. All patients were admitted to the hospital because of a presumptive diagnosis of scarlet fever, and any patient exhibiting borderline or indefinite clinical criteria for this diagnosis was not admitted to the study wards. The patients were largely recruits from the U. S. Naval Training Center, Bainbridge, but a small number of more permanent naval personnel were admitted to these wards with scarlet fever. No patient was admitted to any study group if he had received any antibiotic therapy within two weeks of the onset of his illness.

Clinical Observations.—Each patient had on admission a thorough history and physical examination recorded in the clinical chart. In addition to this and the usual medical records, a marginal punch card and special study forms were completed on entry and followed daily on each patient by the physician. All patients were seen twice daily and examined by the same physician, and more often if necessary. Careful notes were made in the clinical record and on the punch cards as to the persistence of symptoms, physical signs, status of treatment, antibiotic toxicity, and any unusual developments. The oral temperature was also recorded with great regularity on each patient.

Laboratory Observations.—On entry to the study wards each patient routinely had performed a white blood cell count (WBC) with differential smear, a complete urinalysis, and a venesection of 50 ml. for serology. The white blood cell counts were repeated each morning thereafter throughout the period of hospitalization. Urinalyses were repeated on the seventh, tenth, and fourteenth hospital days in all instances, and more often as indicated. On the fourteenth hospital day each patient was again bled of 50 ml. for serologic purposes. Roentgenograms of the lungs were made on admission on all patients and repeated as necessary.

Bacteriologic and Serologic Observations.—Nose and throat cultures were obtained before any treatment regimen was started in every patient and were repeated daily throughout the hospital course. These cultures were obtained under direct vision, with

the aid of a strong white light (a miner's type head lamp was used). A modified Pike technique[19] was used in processing the cultures, the swabs being incubated for four hours at 37° C. in sheep's blood broth and then streaked on to sheep's blood agar plates.

After 18 hours' incubation at 37° C. the agar plates were examined both grossly and with the aid of a hand lens (3×) for the presence of beta hemolytic streptococci. The abundance of growth of such organisms was roughly quantitated from 1 to 3 plus, and representative colonies were picked for serologic grouping and typing by the usual procedure employed in this laboratory, as described elsewhere.[20] The identification of hemolytic streptococci with subsequent grouping and typing of prototype colonies was performed daily on all cultures.

The paired sera from each patient, obtained on entry and on the fourteenth hospital day, were used for serologic measurements. Titers were determined for antistreptolysin 0 in both specimens by a method previously described.[21] Likewise the serial titers for influenzal antibodies were measured by the method of Hirst and Pickels.[22]

Tests for the antibiotic sensitivity of the strains of beta hemolytic streptococci isolated during the study were not routinely performed, so it is neither possible to compare the two agents on this basis nor to ascertain if strains appeared which were resistant to either agent. There is, however, considerable evidence from previous reports suggesting that the drugs are indeed highly comparable in vitro and that resistant strains of group A streptococci are not naturally found.[7]

TREATMENT REGIMENS

In the early part of the study, patients were entered in strict rotation into two treatment schedules: erythromycin and penicillin, in the dosages and durations as outlined below. When the study was in progress and well organized, an additional rotation with a placebo group was included to insure a more accurate comparison of the results. The treatment schedules, then, for the major part of the study are indicated below:

Erythromycin.—Two hundred milligrams were given by mouth on admission and again every four hours thereafter, for a total daily dosage of 1.2 Gm. Treatment was continued for ten full days. No regard was given to timing of doses in relation to meals, and only water was allowed to be taken with each tablet.

Penicillin.—Three hundred thousand units of aqueous crystalline penicillin G were given intramuscularly initially, and then followed by 300,000 units of aqueous procaine penicillin given intramuscularly also every twelve hours. Therapy was continued for ten full days.

Placebo.—Tablets identical in size and color to the erythromycin tablets were given on the same schedule. It was almost impossible for anyone to know whether the patient was receiving the antibiotic or the placebo, inasmuch as the medicines were dispensed by a code number to the ward nurses. Likewise it was virtually impossible for the physician to be biased greatly, for he could not conceivably remember which of the 100 or more patients on the wards at a given time were on either of the two tablet medications. During ward rounds and at the time of recording the various daily observations, no information was available to the physician as to the patient's treatment regimen. All patients receiving the placebos were continued for ten full days, then changed for the remainder of their hospital stay to the penicillin regimen as noted above. This was necessary to reduce the number of patients who would return to duty still harboring streptococci.

In addition to the above treatment groups, all patients received similar supportive and symptomatic therapy. This included bed rest while febrile, adequate fluids, saline gargles, nose drops, elixir of terpin hydrate, and codeine sulfate for relief of headache, chest pain, sore throat, or cough. The routine use of any salicylates or other antipyretic drugs was interdicted in all these patients, because the temperature was used as a criterion

of the degree of illness. An occasional single dose of 0.6 Gm. of an antipyretic, given for a specific indication, was allowed and did not appear to alter the interpretation of the results.

ANALYSIS OF CASES STUDIED

Comparability of Populations.—It is of course almost impossible to obtain exactly comparable populations in the biological experiment. It is possible, however, to obtain groups whose behavior is influenced by roughly similar factors. Because of the fairly rigid physical standards for entrance into the Navy, the well organized daily schedules, and the selection of recruits from the same age groups and geographic origins for a given Navy training center, the population included in this study should offer itself for a sufficiently reliable comparison of the therapeutic efficacy of a drug. In Table I are listed certain aspects of the previous medical history of the patients studied. Although certain differences are noted, it is immediately evident that considering the relatively small number of patients in each group, the incidence of a history of tonsillectomy, allergy, recent upper respiratory infection, scarlet fever, etc., is about equal for all groups. These data are also in agreement with figures obtained on other naval recruits elsewhere.[11]

TABLE I. CERTAIN ASPECTS OF MEDICAL HISTORY IN 208 NAVAL RECRUITS

TREATMENT GROUP	NUMBER OF MEN	PER CENT WITH HISTORY OF					
		T & A*	ALLERGY	RECENT U.R.I.†	RECENT HOSP.‡	SCARLET FEVER	PENICILLIN SENSITIVITY
Erythromycin	78	50.0	17.9	43.6	28.2	5.1	2.6
Penicillin	78	48.7	8.9	32.1	14.1	3.9	1.3
Placebo	52	36.5	19.2	40.4	23.1	3.8	1.9
Total	208	46.2	14.9	38.5	21.6	4.3	1.9

*T & A: Tonsillectomy and adenoidectomy.
†U.R.I.: Upper respiratory infection within one week prior to onset of scarlet fever.
‡HOSP.: Hospitalization for respiratory infection during current winter.

In the following sections further data will be presented that indicate a fairly comparable population existed in all three treatment groups. It is worth noting this information, for many studies are invalidated by failure to realize that marked differences in behavior may occur from group to group. It would not appear to have been a factor in this instance.

Clinical Picture and Diagnostic Features.—In Table II are summarized certain of the more outstanding and pertinent findings from the history and physical examinations of these patients on entry to the wards. Although certain variations did occur from one treatment group to another, in general the incidence of any particular symptom or sign was roughly equivalent in the three regimens. Obviously all patients had the typical scarlatiniform rash. Almost all patients complained of malaise. Chilly sensations were likewise a common finding, with frank, shaking chills occurring quite infrequently. Headache was present in about two-thirds of the patients. Virtually all patients complained of some degree of sore throat on entry, but only about 14 per cent had exudate present in the nasopharynx; 20 per cent had erythema, injection, lymphoid

hyperplasia, or other minor changes; and 65 per cent had no evidence on physical examination of any visible involvement of the throat. The majority of patients also complained of tender cervical nodes of varying degrees, the more markedly tender nodes almost always being associated with the markedly sore throats and the complaint of dysphagia. The incidence of negative examinations of the throat appeared to bear no relationship to the degree of sore throat or tenderness of cervical nodes. Myalgia was noted in almost half of the patients, arthralgia in one-fourth, and nausea and/or vomiting in about one-third of the patients. No patient had the classical "strawberry tongue" on admission, and only 16 per cent of the patients developed this sign at any time during their hospital stay. The incidence was only slightly higher in the placebo treated group, and, because of the very small numbers involved, no possible comparison can be made. Some desquamation, especially of the hands

TABLE II. CLINICAL HISTORY AND FINDINGS IN 208 PATIENTS WITH SCARLET FEVER

SYMPTOM OR SIGN	TREATMENT GROUP						TOTAL	
	ERYTHROMYCIN		PENICILLIN		PLACEBO			
	NO.	%	NO.	%	NO.	%	NO.	%
Malaise	69	88.5	75	96.2	48	92.3	192	92.3
Chilly sensations	61	78.2	73	93.6	32	61.5	166	79.8
Headache	47	60.3	50	64.0	32	61.5	129	62.0
+Sore throat	26	33.3	13	16.7	22	42.3	61	29.3
++Sore throat	49	62.8	61	78.2	28	53.8	138	66.3
+Tender cerv. nodes	42	53.8	22	28.2	23	44.2	87	41.8
++Tender cerv. nodes	12	15.4	20	25.6	11	21.2	43	20.7
Dysphagia	39	50.0	54	69.2	27	51.9	120	57.7
Myalgia	34	43.6	33	42.3	21	40.4	88	42.3
Arthralgia	20	25.6	21	26.9	16	30.8	57	27.4
Nausea and vomiting	27	34.6	34	43.6	15	28.8	76	36.5
Pharyngeal exudate	9	11.5	14	17.9	6	11.5	29	13.9
Throat exam. negative	54	69.2	43	55.1	39	75.0	136	65.4
TOTAL PATIENTS	78	100.0	78	100.0	52	100.0	208	100.0

+ mild to moderate.
++ moderate to severe.

TABLE III. MISCELLANEOUS OBSERVATIONS IN 208 PATIENTS WITH SCARLET FEVER

OBSERVATION	TREATMENT GROUP						TOTAL	
	ERYTHROMYCIN		PENICILLIN		PLACEBO			
	NO.	%	NO.	%	NO.	%	NO.	%
+Throat culture for group A streptococcus	76	97.4	72	92.3	52	100.0	200	96.2
Initial WBC < 10,000	2	2.6	6	7.7	4	7.7	12	5.8
Initial WBC 10-11,900	11	14.1	10	12.8	3	5.7	24	11.6
Initial WBC 12-15,900	28	35.9	18	23.1	14	26.9	60	28.8
Initial WBC 16,000 or >	37	47.4	44	56.4	31	59.6	112	53.8
Initial temp. < 100 F	16	20.5	10	12.8	9	17.3	35	16.8
Initial temp. 100-101.9	35	44.9	35	44.9	25	48.1	95	45.7
Initial temp. 102 or >	27	34.6	33	42.3	18	34.6	78	37.5
Onset 24 hours or <	43	55.1	35	44.9	22	42.3	100	48.1
Onset 25-72 hours	33	42.3	43	55.1	28	53.8	104	50.0
Onset > 72 hours	2	2.6	0	0.0	2	3.8	4	1.9
Serology completed	78	100.0	73	93.6	50	96.2	201	96.6
Rise in Hirst titer	17	21.8	13	16.7	16	30.7	46	22.1
TOTAL PATIENTS	78	100.0	78	100.0	52	100.0	208	100.0

and feet, occurred in most patients, but was severe in only a few. Many of these had underlying chronic epidermophytosis. No suppurative complications were seen in any group.

It also seemed of interest to review the occurrence of some clinical and laboratory observations in relation to the diagnosis of scarlet fever. Some of these findings are shown in Table III. Of the 208 patients studied, 200, or 96 per cent, had on entry nose and/or throat cultures which yielded group A streptococci. One additional patient who was negative on admission had a positive culture on the second hospital day. Of these 201 strains of group A streptococci, all but three were type 19. From two patients type 12 streptococci were recovered, and type 1 from one additional patient.

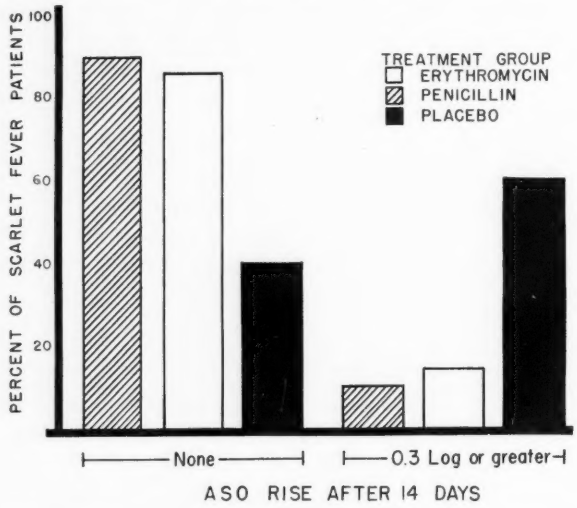

Fig. 1.—Effect of treatment on the antistreptolysin O (ASO) response after 14 days.

The initial white blood cell counts (WBC) are shown in Table III. It is obvious that counts of any order of magnitude may occur and even that some patients exhibited no leukocytosis. It is worth noting, however, that about 83 per cent had counts over 12,000 per cubic millimeter and 54 per cent over 16,000. Similarly, very few patients had an oral temperature below 100° F. on admission; about half fell between 100 and 101.9° F., and 38 per cent were over 102° F. The onset from the first discernible symptom to hospitalization for scarlet fever was generally rapid, with nearly half of the patients seeking medical attention in less than twenty-four hours. It was a rare patient whose illness progressed so slowly or who was so stoical that the onset extended to more than seventy-two hours, and, in fact, further breakdown of the data shows that about 90 per cent had sought medical aid within forty-eight hours.

Serology.—Serial antistreptolysin O (ASO) determinations were completed in 201 (97 per cent) of the cases. Fig. 1 shows these results graphically. When a rise in titer of 0.3 log (3 tubes) was considered significant, it was found that only 14 per cent of the erythromycin treated group and 10 per cent of the

penicillin treated group had such increases in titer, in contrast to the placebo group with rises in 60 per cent of the patients. If a rise of 0.2 log was considered significant, then the figures became 28 per cent for the erythromycin, 24 per cent for the penicillin, and 83 per cent for the placebo treated groups.

Because of a superimposed explosive outbreak of epidemic influenza, due to an A prime virus, it seemed of interest to subject these sera to determinations for antihemagglutinins against PR 8, FM 1, Lee, and the 1953 prototype virus strains. Significant rises in titer occurred in 22 per cent of the erythromycin, 17 per cent of the penicillin, and 31 per cent of the placebo treated patients. When these cases were excluded from the analysis of the clinical and diagnostic features and from the analysis of the results of the three therapeutic regimens, no significant differences were found.

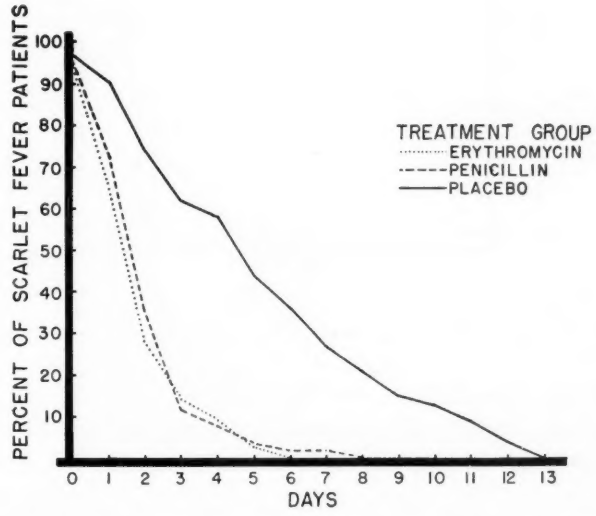

Fig. 2.—Effect of treatment on the duration of fever in patients with scarlet fever.

RESULTS

The analysis of the effect of each of the three treatment regimens on the major aspects of the clinical course of 208 patients with scarlet fever is graphically illustrated in Figs. 2 to 6. As nearly as possible the comparison was made on somewhat objective criteria, i.e., clinical or laboratory observations not subject to the personal interpretation of either the patient or the physician.

Effect on the Duration of Fever (Fig. 2).—Arbitrarily fever was defined as an oral temperature of 99.5° F. or above. The maximum oral temperature for each patient was recorded daily and fever was said to persist until a sustained temperature of 99.4° F. or less was achieved. It is evident that a few patients were afebrile even on admission. The curves for the erythromycin and penicillin treated groups are identical in every respect, with more than two-thirds of the patients becoming afebrile by the third hospital day. In contrast, the placebo

group did not achieve a similar percentage of patients becoming afebrile until the sixth day. None of the treated patients were febrile for more than seven days, but a few in the control group had fever for as long as twelve days. It might be added parenthetically that the fall in temperature was accompanied in most instances by evidence of appreciable improvement in the clinical status.

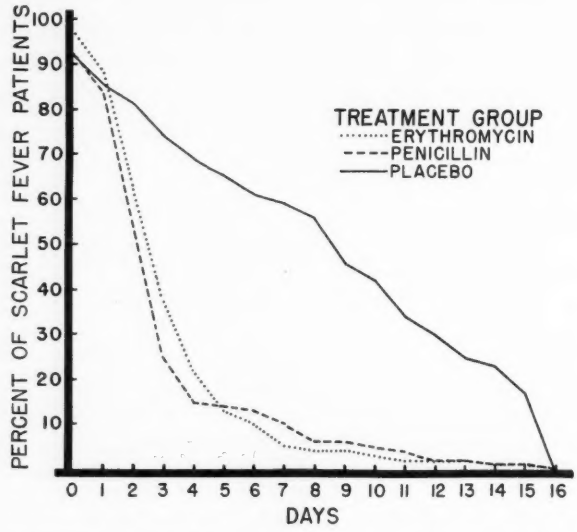

Fig. 3.—Effect of treatment on the duration of leukocytosis associated with scarlet fever.

Effect on the Duration of Luekocytosis (Fig. 3).—Leukocytosis was adjudged present as long as the daily white blood cell count did not maintain a sustained level below 10,000 per cubic millimeter. The incidence of initial counts above and below 10,000 has been shown in Table III. Most of the patients had an elevated leukocyte count on entry, and those who received either erythromycin or penicillin showed a rapid decline, with almost 70 per cent having a normal count on the third hospital day. Again in marked contrast is the placebo group, in which the incidence of leukocytosis did not achieve this level until the twelfth hospital day. A few patients in all groups persisted in having elevated counts throughout most of their hospital stay. It was not possible to correlate these instances of prolonged leukocytosis with any definite clinical phenomena, even in the placebo group. In general, the leukocyte count fell as did the temperature, although perhaps slightly slower. The curves depicted in Figs. 2 and 3 are nearly superimposable.

Effect on the Duration of the Rash (Fig. 4).—Inasmuch as a typical scarlatiniform rash was the sine qua non for admission to the study wards, it was possible to compare its persistence in all patients. There were a few patients each in the erythromycin and penicillin regimens in whom the rash persisted

only twenty-four hours, in spite of the persistence of other signs and symptoms and positive cultures for type 19 streptococci. Generally, the rash faded rapidly and was no longer evident in about 90 per cent of the antibiotic treated patients on the fourth day, whereas the control group had continued evidence of the typical rash for forty-eight hours longer. The rash was present in no instance for more than six days.

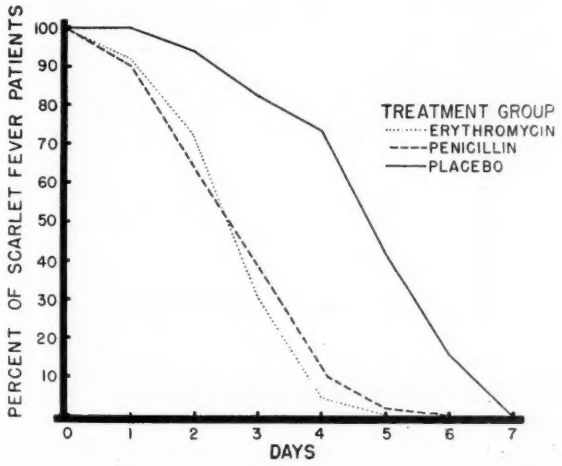

Fig. 4.—Effect of treatment on the duration of the rash in scarlet fever patients.

Effect on the Presence of Group A Beta Hemolytic Streptococci in Nose and Throat Cultures (Fig. 5).—Since only three of the initial 201 strains of group A streptococci recovered from these patients were *not* type 19, no attempt has been made to differentiate the follow-up cultures on the basis of serologic type. It might be stated that in only a rare instance was a later culture shown to have a streptococcus of a different type, and in each of these circumstances another subsequent culture had the original type present again.

The abrupt disappearance of group A streptococci from the cultures of the nose and throat while treatment is continued is one of the most striking findings. The curves for erythromycin and penicillin in this regard are indistinguishable. After only one day of therapy about 95 per cent of the originally positive cultures had reverted to negative, and after three days of therapy with either agent, no positive cultures were obtained as long as treatment was maintained. Following discontinuance of erythromycin after ten days, four patients had recurrences of single positive cultures: one each on the fourteenth and sixteenth days and two on the fifteenth day. After penicillin was stopped, there were five recurrences of positive cultures: one each on the twelfth, thirteenth, and fifteenth days, and two on the fourteenth day. The average hospital stay was sixteen days; thus each man was observed for an

average of six days following cessation of treatment. Follow-up cultures on the twenty-first day yielded no further positives. In all instances the recurrent positive cultures were type 19 streptococci.

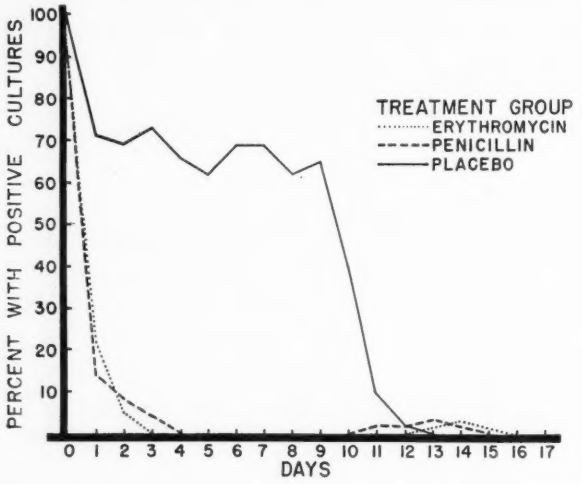

Fig. 5.—Effect of treatment on the persistence of group A beta hemolytic streptococci in cultures from scarlet fever patients.

Fig. 5 also shows the results in the placebo group. It is immediately evident that within forty-eight hours on no specific therapy, nearly thirty per cent of these patients became negative for group A streptococci. During the subsequent eight days no significant change occurred in the number of positive cultures. It should be pointed out again here that after ten days on placebos all these patients were transferred to the penicillin schedule for the remainder of their hospital stay. The marked decline, then, in the number of positive cultures after the tenth day really represents another effect of penicillin. No positive cultures were observed in this latter group after thirteen days, but the patients remained on penicillin until discharge on about the sixteenth day. There were no positive cultures in the follow-up at twenty-one days.

Effect on the Total Duration of Illness (Fig. 6).—The comparison of three regimens on this criterion is the only one based upon somewhat more arbitrary and subjective grounds. The total duration of illness was calculated from the onset of the first symptom associated with the present illness to that time when, in the opinion of the physician, the patient was clinically well. In determining this state of well-being, fever, leukocytosis, cultures, etc., were not considered; this was based solely on examination and critical appraisal of the patient and how he felt. Inasmuch as all patients were treated alike, the comparison is probably justified.

The most striking thing at first glance is that the difference between the erythromycin-penicillin curves and the placebo curve is much less marked than in former observations (Figs. 2 to 5). Over 90 per cent of the antibiotic treated

patients were well after six days, whereas the placebo group required eleven days to reach the same point. A few patients in all groups required slightly longer to recover, but no one required more than fourteen days from the onset of the first symptom.

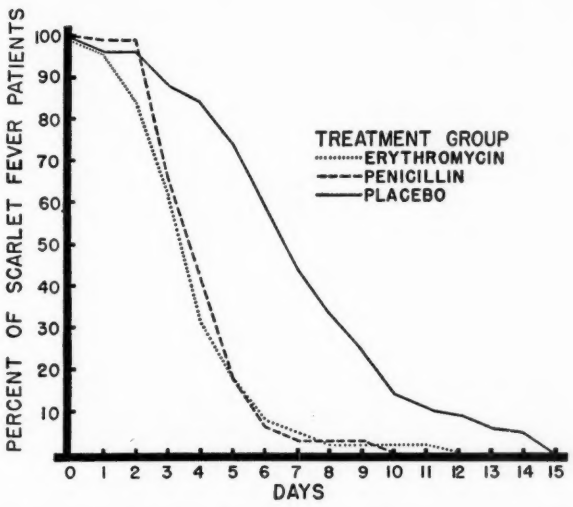

Fig. 6.—Effect of treatment on the total duration of the illness in patients with scarlet fever.

Toxicity.—A majority of the patients had never received any antibiotic therapy and were unaware of any potential toxicity. No attempt was made to instruct them in reporting any signs or symptoms. As shown in Table I about 2 per cent alleged to have a history of sensitivity to penicillin, but patients were assigned in strict rotation to the three therapeutic regimens without regard to the medical history. Since all patients were seen at least twice daily by the same physician, it was felt that any symptoms would be voluntarily reported and any physical signs of toxicity noted. In those receiving erythromycin only three toxic reactions were noted: two patients complained of vague upper abdominal distress, some hypermotility of the bowels, and slightly loose and bulky stools; one patient developed frank but not severe watery diarrhea, which subsided without specific treatment and without missing a dose of medication. Twelve patients in the penicillin group had to be classified as having toxic reactions. In each instance the rash or urticaria was definitely distinguishable from the scarlatiniform rash. All these reactions occurred while the patient was receiving penicillin, usually began about the fifth day, were generally well controlled with antihistamines, and in no instance required the abrogation of the antibiotic schedule. One patient developed massive giant urticaria and it was necessary to control this with Adrenalin; it is of interest to note that he had a previous history of penicillin sensitivity. Reactions were also observed in the placebo group: one patient developed mild urticaria while receiving the inert tablets, but it subsided with antihistamines; one other patient developed

a similar gastrointestinal syndrome to the erythromycin treated patients, including frank diarrhea. Of greatest interest is the fact that eight of the placebo treated patients who later received penicillin before discharge developed similar rashes and urticaria in this latter period. The lesions were identical in type, appeared after four or five days on penicillin, subsided well with antihistamines, and did not require cessation of penicillin.

DISCUSSION

The data presented above offer further convincing evidence of the effectiveness of erythromycin against the hemolytic streptococcus, and in particular in the treatment of scarlet fever. This is shown clearly by the prompt subsidence of fever, the rapid decline in leukocyte counts, the abrupt disappearance of group A streptococci from cultures of the nose and throat, and the abbreviation of the total duration of illness. That these effects would not have been obtained otherwise is demonstrated by comparison with the placebo group of patients. Although the number of patients in any one group is not large, the differences are so marked that statistical manipulations are quite unnecessary to prove their validity.

Further the comparison of the results obtained in the erythromycin and penicillin treated groups leaves little to the imagination. On all criteria selected it is virtually impossible to distinguish the effects of the two antibiotics. In fact, the similarity is remarkable, and the graphic data shown in Figs. 2 to 6 are almost superimposable on each other. Erythromycin given by mouth in average doses and without relation to meals or adjuvant feeding was as effective as a standard regimen of procaine penicillin given intramuscularly. No attempt was made to determine if smaller quantities of either antibiotic would have been as effective; sufficient data exist in the literature to prove that considerably smaller daily dosages have produced similar results.[2, 16] Furthermore, it was not feasible to compare identical quantities of erythromycin and penicillin given orally and at random. This latter point would be of great interest, in view of increasing evidence that oral penicillin in adequate amounts is highly effective. No good data are available at present on the comparative efficacy of the two oral preparations given on a weight basis.

Certain other points are worthy of mention. Although the results within the study are probably comparable, it may not be fair to compare this study population with other studies. These were all young males in their peak of physical condition and health. They had immediately available medical assistance and were generally hospitalized soon after the onset of the illness. This may account, for example, for the rather low incidence of the finding of "strawberry tongue." The classical triad of rash, nausea and vomiting, and strawberry tongue was seldom seen; and indeed the main symptoms and signs were generally those of acute infection of any type. The common concomitants of any streptococcal infection were also evident, and the correlation was similar to that shown by others.[23] Patients with leukocyte counts above 12,000 per cubic mil-

limeter, temperatures above 100° F., tender cervical nodes, and positive cultures for hemolytic streptococci comprised the bulk of this group of scarlet fever patients.

The finding of completely negative-appearing throats in spite of the high symptomatic rate is of interest. Often the so-called "red throat" is in fact due to a poor or too yellow light. It has been found that the routine use of a strong white light obviates this artefact in physical diagnosis. This unexpected part of the clinical picture may be related perhaps to the abrupt onset of the illness, the short period between onset and hospitalization, and the fact that often the rash takes the patient to a physician long before any symptoms might have sent him there. The relatively low incidence of exudative pharyngitis in these patients lend credence to the viewpoint that scarlet fever is mainly a systemic disease and the throat is only transiently involved as a portal of entry. The presence of tonsils in these patients was about the same as noted elsewhere,[11] so the lack of exudate is not explainable on that basis.

The serologic data demonstrate again that the administration of an antibiotic in the early stages of a streptococcal infection may inhibit the formation of antistreptolysin O. These figures show that erythromycin behaves in this manner similarly to penicillin.[24-26] The placebo group exhibited significant rises in ASO titers in about as many instances as reported by others.[23, 27] The hope that an antibiotic might possess the powerful bactericidal and curative powers of penicillin and yet allow the human body to develop its own type-specific protective antibodies has not been answered with erythromycin.

It is worthy of mention in passing that during an explosive A prime influenza epidemic on the base, there was an incidence of influenza of about 22 per cent (diagnosed by rises in antibody titer) in patients hospitalized for scarlet fever. Whether one illness masked the other, or whether these changes in titer reflect truly subclinical influenzal infections would be impossible to determine. It was evident from analysis of the data, however, that the exclusion of these patients did not significantly alter the percentages for any criteria. One might speculate further on the number of placebo treated patients exhibiting rises in influenzal antibodies as compared to the two antibiotic treated groups. Because of the small numbers involved, no conclusions may be drawn; but it is worth speculating on the possible influence of these antibiotics on the formation of this antibody.

The slightly longer duration of the rash in those patients who did not receive antibiotic therapy suggests that the rapid bactericidal action of the two agents diminishes the amount of erythrogenic toxin absorbed and thus abbreviates the duration of the rash. If this is the complete explanation, however, one might wonder why the difference is not greater, for at ten days about 70 per cent of the placebo group are still showing positive cultures. Critical studies on in vivo production of antitoxin in relation to therapy and time would have to be made to determine the relative roles of the antibiotic and host factors in the persistence of the rash.

The bacteriologic data show that ten days of adequate therapy with either erythromycin or penicillin is largely successful in eradicating the streptococcus

from the nose and throat. There were few relapses, and all of these were only single isolations. It would be difficult to classify these as carriers, parasitisms, or reinfections in view of the continued prevalence of the type 19 streptococcus on the base at that time.

The only marked difference between the two antibiotics was in toxicity. While it was impossible to follow all the patients for prolonged periods and check them for persistent penicillin sensitivity, this would not necessarily have established the rash or urticaria as penicillin toxicity. It is generally known that even following definite penicillin reactions, a skin test or therapeutic test may be negative. Inasmuch as these skin manifestations occurred while on penicillin, were controlled by conventional therapy, and did not recur after omission of antihistamines and penicillin, it seems fair to call them penicillin reactions. This incidence of 15 per cent is in striking contrast to the 4 per cent in the erythromycin and 4 per cent in the placebo groups. Furthermore, 15 per cent reactions also occurred when the placebo group was placed on penicillin after ten days.

Complete comparisons of these results with those of others are difficult to make and are not justified. For one thing, most of the preliminary studies have employed erythromycin in the treatment of specific infections with various dosage forms and schedules. This study is primarily a controlled observation on the comparative efficacy of the two drugs, erythromycin and penicillin. Penicillin had long ago been shown to be highly effective in treating scarlet fever, in suppressing suppurative complications, and in reducing later complications such as rheumatic fever and nephritis.[13, 17] The present data suggest that erythromycin is equally effective on all scores.

SUMMARY AND CONCLUSIONS

Clinical, bacteriologic, and serologic findings in 208 patients with scarlet fever have been presented. Three treatment regimens were used in strict rotation: erythromycin, penicillin, and placebo.

Detailed data are given on the clinical picture and diagnostic features of scarlet fever as seen in Naval personnel, with notes on why this picture may vary from the general population.

Using the effect of the two antibiotics on the objective criteria of the duration of fever, the duration of leukocytosis, the duration of the rash, the persistence of group A beta hemolytic streptococci in cultures, and the total duration of illness, no significant differences were found between erythromycin and procaine penicillin. These two antibiotics exhibited activity in marked contrast to the placebo treated group.

Erythromycin largely suppressed the formation of antistreptolysin O as did penicillin. This was not true of the placebo group.

A significant number of those patients receiving penicillin either initially or following placebo therapy developed rashes or urticaria that had to be ascribed to penicillin. Two patients receiving erythromycin developed upper

abdominal distress and hypermotility of the bowels; another patient had frank watery diarrhea. One patient receiving placebos developed a mild urticaria, and another had a similar upper gastrointestinal syndrome to the erythromycin treated patients.

Erythromycin is apparently as effective in the treatment of scarlet fever and in the prevention of its suppurative complications as is procaine penicillin. The incidence of toxicity was significantly less in the erythromycin treated patients.

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TYPE C INFLUENZA VIRUS

I. Studies of the Virus and Its Distribution

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PREVIOUS reports from this laboratory[1, 2] described a new type of virus (JJ) occurring concurrently with an outbreak of influenza A-prime and a note on some of the experimental work carried out with this strain. The virus was shown to be essentially the same as strain 1233 isolated by Taylor[3] in 1947. From the clinical data available the agent caused an illness indistinguishable from mild influenza. Furthermore, in the local outbreak where antibody rises to it were first noted in a large proportion of sera, it appeared to be as prevalent as the A-prime infection. For these reasons, and because of other immunological data, it was suggested that the disease be called influenza C.

Many of the observations were merely summarized in our original paper[1] and have been substantiated in a later report by Taylor.[4] The present paper gives details of the isolation of the virus, its characteristics, attempts to adapt it to experimental animals, and serologic studies.

MATERIALS AND METHODS

Throat Washings.—Throat washings were obtained from individuals ill with influenza-like disease within the first four days of onset of illness. The washings were collected by having the patient gargle vigorously two or three times with small amounts of beef extract broth, pooling the garglings. Six hundred units of penicillin and 1.25 mg. of streptomycin per milliliter were added to the throat washings before inoculation into eggs.

Amniotic Inoculation of Chick Embryos.—The method of amniotic inoculation was the window technique of Beveridge and Burnet.[5] One-tenth milliliter of inoculum was injected directly into the amniotic cavity of 10-day-old embryonate eggs, and the window sealed with cellulose tape. The eggs were incubated at 35° C. for three days (later reduced to two days). After removing the allantoic fluid and chorioallantoic membrane, the amnion was picked up with forceps and the fluid withdrawn with a syringe and needle. On some occasions the yield of amniotic fluid was very small and it was necessary to wash the cavity with 2 ml. of sterile normal saline.

Viruses.—The JJ[1] and 1233[3] strains of Type C influenza virus were maintained in eggs amniotically. The PR8,[6] Rhodes,[7] and FM1[8] strains of Type A and A-prime viruses and the Lee[9] strain of Type B virus were the influenza strains used for comparative purposes.

Cells.—Blood from human subjects or various species of animals was drawn into a solution of 5 per cent sodium citrate in saline and placed in a 50 ml. graduated centrifuge tube. The cells were washed three times and the packed cells made up to 10 per cent by volume in sterile saline for storage. Subsequent cell suspensions were made from this stock. No cells over 6 days old were used.

Titration of Hemagglutination.—Starting with a 1/20 dilution of amniotic fluid, two-fold serial dilutions were made in 0.5 ml. of saline, using a separate pipette to mix each

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dilution; 0.5 ml. of either 0.5 per cent or 1 per cent red blood cells were added and the tubes shaken. Unless otherwise stated, all hemagglutination tests with Type C influenza virus were kept at 6° C. for 1½ to 2 hours and then read on the basis of cell patterns. The titers are expressed as reciprocals of the initial dilution of the virus.

Animals.—Both albino and brown ferrets approximately 1 year old were used for antiserum and attempted adaptation of the virus. In attempts to adapt the virus to other laboratory animals both suckling and 4-week-old mice of the Webster strain and hamsters, approximately 50 to 100 grams in weight, were employed. Six rhesus monkeys previously inoculated with poliomyelitis virus were inoculated by various routes.

Sera.—Sixty-eight sets of sera covering the interval of time from August, 1946, through May, 1947. were obtained from children residing in an institution who had been vaccinated against the PR8 strain of Type A influenza virus.[10] Fifteen pairs of acute and convalescent specimens were also obtained from adults in this children's home who were ill during an outbreak which had been thought to be primarily due to Type A-prime strains of influenza virus.

Samples of acute and convalescent sera from other Type A, A-prime, and Type B epidemic periods, and pre- and postinfluenza vaccination sera, were examined, as shown in the accompanying table.

NO. OF SAMPLES	YEAR	TYPE
9	1936	A
5	1940	A
23	1945	B
53	1947	A′
41	1950	A′
215	1951	A′
133	1952	B
6	1948	Vaccinated with A′ (FM1 only) influenza virus
25	1952	Vaccinated with polyvalent A (PR8, FM1, Cuppett)
26	1952	Vaccinated with Type B (Lee)

The pairs of sera shown in the second of these two tables represent illness other than influenza.

NO. OF SAMPLES	YEAR	DIAGNOSIS
77	1950	Acute respiratory*
6	1949-50	Common cold†
7	1947-50	Atypical pneumonia†
18	1949-50	Infectious mononucleosis‡
3	1950	Rubeola
7	1950	Rubella
26	1947	Mild poliomyelitis

*Including samples obtained from Dr. J. E. Salk, of the University of Pittsburgh, and samples obtained from Dr. Meiklejohn, of the University of California.

†Obtained from Dr. A. E. Feller of Western Reserve University.

‡Obtained from Dr. C. Zarafonetis of the University of Michigan.

Ferret sera used in testing cross serologic reactions were obtained before and after intranasal infection, the rabbit and mouse sera after intraperitoneal injections of the specified virus. All sera were stored at refrigerator temperature in rubber-stoppered test tubes.

Hemagglutination-Inhibition Test.—The method employed was that recommended by the Committee on Standard Serological Procedures in Influenza Studies[11] with some modifications. All serum titers are expressed as reciprocals of final dilutions (including antigen and cells) instead of initial dilutions. Because the hemagglutination of JJ and 1233 strains of virus is more consistent and reaches higher titers at 4 to 6° C., incubation of tests with these strains was carried out in the cold for 1½ to 2 hours before readings were made.

Complement-Fixation Tests.—The diluent used was 0.85 per cent sodium chloride solution containing 0.01 per cent magnesium sulfate. Sheep erythrocytes were sensitized by rapidly mixing equal parts of 2.5 per cent cells and hemolysin. The latter was diluted so that two units were contained in 0.2 ml. Fresh complement was used in a concentration of two units in 0.2 ml. The unit of complement was obtained by titering in the presence of antigen. The unit of antigen (infected amniotic or allantoic fluid) was obtained by titering serial twofold dilutions of antigens with serial twofold dilutions of immune animal serum. The highest dilution of antigen showing 3+ fixation with the highest dilution of immune serum was regarded as 1 unit. The antigen was diluted to contain 2 units in 0.2 ml. The sera were inactivated at 56° C. for 30 minutes and diluted in twofold steps; 0.2 ml. each of serum dilution, antigen and complement were mixed and incubated at 37° C. for one hour, and the sensitized sheep cells were then added in 0.5 ml. amounts. The test was read after further incubation for 30 minutes in a 37° C. water bath. Titers are expressed as the reciprocal of the highest initial dilution of serum that gave a 4+ fixation of complement.

In Ovo Neutralization Test.—All sera were heated at 56° C. for 30 minutes and diluted in fourfold steps in 0.85 per cent solution of sodium chloride containing 10 per cent normal horse serum. Dilutions of virus were made in the same diluent. Equal volumes of serum dilution and of the virus preparation containing 100 to 1,000 egg infectious doses were mixed and incubated at 37° C. for 30 minutes. One-tenth milliliter of each dilution of serum-virus mixture was inoculated into the amniotic sac of four to six embryonate eggs (10 days old) when testing for neutralization of the JJ or 1233 strains or via the allantoic cavity when testing for the neutralization of the other strains of influenza virus. After incubation at 35° C. for 96 hours, fluid, either amniotic or allantoic, was removed from each egg and tested separately for hemagglutination with chicken red blood cells. The neutralization titer was recorded as the 50 per cent end point calculated by the method of Reed and Muench.[12]

EXPERIMENTAL

Isolation of Virus.—The initial isolation of the JJ strain of Type C influenza virus was accomplished by amniotic inoculation of eggs with garglings obtained a few hours earlier. It was, however, subsequently observed that such garglings could be stored at 6° C. for as long as four days and still yield virus, while one known positive throat washing was negative when retested after storage in the CO_2 ice chamber for three days. Taylor has stated that virus was isolated after storage of throat washings in CO_2 icebox, but the time is not given.

In the interval from the middle of March, 1950, until April, 1951, approximately 125 individual throat garglings were examined for the presence of Type C influenza virus. The majority had been obtained from adults ill during outbreaks of A-prime influenza, although a few were from cases of influenza-like disease occurring between the epidemic in the spring of 1950 and that in the late winter and spring of 1951. During that time, Type C virus was recovered from one case in 1950 (JJ) and another in 1951 (BP).[13]

The growth characteristics of the JJ strain were similar to those described by Taylor[3, 4] and Hirst[14] for 1233. The amount of virus produced in the allantoic sac of 10-day-old embryonate eggs, as determined by hemagglutination, was very erratic and of low titer, ranging from less than 1:2 to 1:256, while growth in the amnion produced high concentrations of virus with hemagglutination titers of 1:2048 to 1:4096 and infectivity titers between 10^{-9} and 10^{-10}. It is also worth mentioning, as previously,[1] that no poxlike lesions developed on the embryo or its membranes even with prolonged incubation, nor

were there any LCL bodies or other types of inclusions demonstrated. However, Taylor states that Johnson had observed cytoplasmic inclusions in the amniotic membrane of infected embryos.[4]

Hemagglutination.—The hemagglutinating characteristics of the JJ and 1233 strains of Type C influenza virus with erythrocytes of various species at 24° C. and 6° C. are shown in a typical experiment in Table I. The best titers were obtained with chicken, hamster, and human cells at 6° C. It has been noticed, however, that the hemagglutination titer of a single sample of virus may vary from less than 1:10 to 1:640 with human cells from different individuals. Further investigation of this phenomenon is in progress. As yet, it does not seem to be a simple function of the various blood groups. Mouse cells were the only ones to show consistently higher agglutination titers at 24° C. The lack of hemagglutination with guinea pig cells serves as a very important guide in distinguishing the Type C from the other types of influenza virus. Although Taylor states[4] that agglutination of guinea pig cells was observed with early passages of the 1233 strain, it has never been observed in this laboratory.

TABLE I. HEMAGGLUTINATION OF JJ AND 1233 STRAINS OF INFLUENZA C VIRUS AT 6° C. AND 24° C. WITH ERYTHROCYTES OF DIFFERENT ANIMAL SPECIES

ERYTHROCYTES	JJ		1233	
	6° C.	24° C.	6° C.	24° C.
1% chicken	640	<20	320	20
1% human "O" (E. M.)	160	<20	160	<20
1% guinea pig	<20	<20	<20	<20
1% hamster	320	20	320	80
1% monkey	<20	<20	<20	<20
1% ferret	<20	<20	<20	<20
1% mouse	40	160	40	160
1% sheep	<20	<20	<20	<20

When heated at 56° C. for 10 minutes or more, infectivity of the JJ virus was lost, but the hemagglutinin remained almost unchanged even after 60 minutes.

Adsorption and Elution.—The capacity of the JJ strain to adsorb and elute from chicken red cells is strikingly different from the other types of influenza viruses. When equal volumes of undiluted allantoic fluid of either Types A, A-prime, or B influenza viruses and 2 per cent RBC are mixed at 6° C. for one hour, the supernatant fluids reveal a marked loss of hemagglutinin, and the eluates from the adsorbed cells, in fresh saline, have hemagglutination titers essentially equivalent to those of the original allantoic fluids. However, when the JJ strain is treated in the same manner, there is no decrease in hemagglutinin of the supernate and only a minimal amount appears in the eluate. Even when the concentration of RBC is increased from 2 per cent to 10 per cent, there is no decrease of hemagglutinin in the supernatant fluid, and there is only a slight amount in the eluate. Apparently not more than 0.1 per cent of virus is adsorbed (Table II). Furthermore, virus heated at 56° C. so as to destroy its infectivity does not become fixed to the RBC.

TABLE II. HEMAGGLUTINATION AND INFECTIVITY RESULTS OF ADSORPTION AND ELUTION WITH JJ STRAIN

SPECIMEN	HEMAGGLUTINATION TITER ACTIVE JJ	HEMAGGLUTINATION TITER INACTIVE JJ	INFECTIVITY TITER ACTIVE JJ
Amniotic fluid control	1556	1024	$10^{-9.66}$
Supernate after adsorption (10% cells at 6° C.)	1556	1024	10^{-10}
Eluate*	12	16	$10^{-6.75}$

*After washing adsorbed cells three times in saline, they were eluted at 37° C. for 2 hours.

Subsequently, it was found that some adsorption of the virus could be demonstrated with repeated additions of RBC to diluted amniotic fluid. This is illustrated in the following experiment: 2 ml. of packed RBC were mixed with 20 ml. of JJ amniotic fluid diluted 1/10 in saline and placed in the cold room at 6° C. for 15 minutes. The cells were centrifuged out while still at 6° C. and the supernate was removed. A sample of this supernate was saved for other tests and the remainder was again adsorbed with fresh packed cells in a ratio of 1 part RBC to 10 parts supernatant fluid. This procedure was carried out for a total of six adsorptions. Agglutination was visible in the first three adsorptions and in the fourth there appeared to be slight clumping at the bottom of the tube, but no visible agglutination could be detected in the fifth and sixth adsorptions. Samples of the original fluid and each of the six supernatant fluids were titrated for hemagglutinins, and the original and supernates 2, 4, and 6 were titrated in eggs, amniotically, for infectivity (Table III).

TABLE III. HEMAGGLUTINATION AND EGG INFECTIOUS TITERS OF JJ AMNIOTIC FLUID AFTER REPEATED ADSORPTIONS WITH CHICKEN RBC

SPECIMEN	H. TITER	E.I. TITER
Original Fluid	256	$10^{-8.17}$
1st Supernate	256	
2nd Supernate	32	$10^{-6.23}$
3rd Supernate	0	
4th Supernate	0	$10^{-5.0}$
5th Supernate	0	
6th Supernate	0	$10^{-4.23}$

Again, the hemagglutination titer of the first supernatant fluid is identical with the starting material, but from the second adsorption on, the titers in the supernates dropped off rapidly. The egg infectivities of the supernates, likewise, declined appreciably. No explanation is at hand of the failure to obtain significant adsorption in a single exposure.

Animal Experiments.—Our experience with intranasal infection of ferrets, hamsters, mice, and monkeys is similar to Taylor's in that all evoked a specific rise in hemagglutination-inhibition antibodies to the JJ strain of virus but no definite signs of illness were noted. Attempts to establish the virus in ferrets and hamsters have met with some success, but all attempts in mice have failed.

Young *ferrets* were inoculated intranasally with 2 ml. of amniotic fluid from the twenty-second egg passage of the JJ virus, containing 10^5 egg infec-

tious units. The ferrets were observed and temperatures recorded twice daily. The animals were sacrificed after three or four days, the turbinates (occasionally portions of lung and trachea) were ground with alundum and made up to a 10 per cent suspension with 10 per cent horse serum in saline containing penicillin and streptomycin; 2 ml. of this suspension were administered intranasally to other ferrets. The amount of virus in the ferret tissue was titrated by inoculating tenfold dilutions of the passage material amniotically into groups of four eggs each. None of the ferrets ever showed evidence of illness by loss of appetite or fever. In the first four serial passages the turbinates appeared to be very red and slightly soft, while the lungs presented no gross lesions. By the fifth passage definite plum-colored lesions appeared in the lungs, and virus was recovered for the first time from the lungs in small amounts, as well as from the turbinates, by amniotic inoculations of eggs. The virus from these eggs was identified as the JJ strain by hemagglutination-inhibition test with type specific antisera. However, in the sixth passage the lungs were again negative and no virus was recovered, but the turbinates were positive. The amount of virus recovered from the turbinates was consistently in the neighborhood of 10^5 egg infectious doses. It is interesting to note that when the infecting dose was reduced to 10^3 egg infectious doses, no virus was recovered from either turbinates or lungs. A 10 per cent suspension of turbinates and lung from the sixth ferret passage was frozen and dried in vacuo. Later, virus was recovered from this lyophilized material in eggs but not in ferrets.

A second attempt to establish the virus in ferrets was undertaken. Three animals were inoculated intranasally with 10^4, 10^6, and 10^9 egg infectious doses, respectively. In subsequent passages 1 ml. of 10 per cent turbinates plus 1 ml. of 20 per cent lung and trachea suspension were passed to the next ferret in each series. In the series initiated with 10^4 egg doses, virus was recovered from the turbinates only in the first and second passages, becoming negative in the third. The second series, starting with 10^6 egg doses, remained positive through three successive passages, while virus was recovered from both the turbinates and lungs of the fourth passage animal in the last series. Unfortunately, the turbinates and lung from this fourth passage were heavily contaminated with a small, penicillin- and streptomycin-resistant gram-negative rod which was indigenous to the ferrets. The suspension to be used for the fifth passage was filtered through a Mandler candle. It contained 10^3 egg infectious doses and was not infectious for the fifth ferret in the series.

The infection in *hamsters* resembles, very closely, the picture in ferrets. Two hamsters, approximately 100 grams in weight, were inoculated intranasally with 0.5 ml. of JJ virus from the thirty-sixth amniotic passage containing 10^6 egg infectious doses to initiate the serial passages. The animals were sacrificed on the third or fourth day and the lungs and turbinates removed. The turbinates were pooled and ground with sufficient beef extract broth containing penicillin and streptomycin to make a 10 per cent suspension. Similarly, the lungs were pooled and made up to 20 per cent suspension. Turbinate suspension, 0.25 ml., and lung suspension, 0.1 ml., were administered

to the next pair of animals. Each suspension was titrated separately in eggs for the presence of virus. Taylor reports[4] that he was successful in making only three serial passages in hamsters, but to date the virus has been maintained through 34 serial passages in this laboratory. The hamsters have shown no signs of illness except in the twenty-third passage when one animal was found dead on the fourth day, 2 sick, and 3 with no signs. At autopsy the lungs have been entirely free of gross lesions with the exception of the thirtieth passage when the lungs of two out of three animals showed small but definite involvement. The turbinates have been consistently soft, red, and moist. Only nine times (passages 2, 7, 10, 11, 15, 16, 18, 29, and 30) has any trace of virus been found in the lungs, but the turbinates have had infectious titers in eggs between 10^{-5} and 10^{-7}.

The twenty-third and twenty-fifth passages were titrated in hamsters (Table IV). Virus was recovered from the 10^{-4} dilution of turbinates but not from the 10^{-5}. Also on the twenty-fifth passage, groups of animals were sacrificed at 6, 8, and 12 days to test the survival of the virus (Table IV). Even after 12 days, virus was recovered from the turbinates in low titer.

TABLE IV. TITRATION AND SURVIVAL OF JJ STRAIN OF INFLUENZA C IN HAMSTERS

DATE	PASS. NO.	DIL. OF INOCULUM		REMARKS	RECOVERY OF VIRUS IN EGGS	
		LUNG	TURB.		LUNG	TURB.
10-30-52	H23	20%	10^{-1}	Hamster 1. No symptoms	0	$10^{-7.3}$
				2. Sick	0	$10^{-7.0}$
		2%	10^{-2}	Hamster 1. Sick	0	$10^{-6.0}$
				2. Dead	0	$10^{-6.3}$
		0.2%	10^{-3}	Hamster 1. No symptoms } pooled 2. No symptoms }	0	$10^{-7.0}$
11-6-52	H25	10%	10^{-3}	Pool	0	$10^{-5.0}$
		10%	10^{-4}	Pool	0	$10^{-5.0}$
		10%	10^{-5}	Pool	0	0
		20%	10^{-1}	Killed 6 days after inoc.	0	$10^{-7.0}$
		20%	10^{-1}	Killed 8 days after inoc.	0	$10^{-3.0}$
		20%	10^{-1}	Killed 12 days after inoc.	0	$10^{-3.0}$

Twice during the course of these experiments resistant hamsters were encountered. The first time the animals were from a different source, but the second time they were from our regular breeder. In each instance, on repeated attempts, virus was maintained for one passage with a drop in egg infectious titer, and after the second passage no virus could be recovered. No explanation has been found to account for this phenomenon. The use of cortisone intramuscularly appeared to have no effect on the susceptibility of these resistant animals.

Seven attempts have been made to adapt the JJ strain of virus to *mice*. Groups of suckling and 4-week-old mice were given intranasally, in 0.05 ml. amounts, approximately 10^9 egg infectious units of virus. Subsequent passages of 10 per cent lung suspensions were made from animals sacrificed one to four days after inoculation. These suspensions were also inoculated into eggs to detect the presence of virus. No virus could be recovered from mouse lungs

beyond the fourth day of the first mouse passage. Furthermore, mice receiving the second and third passages of mouse lung suspensions did not develop antibodies. The addition of *H. influenzae* to the inoculum did not favorably influence these findings. Positive turbinates from both ferrets and hamsters were inoculated intranasally into mice and failed to produce virus even in the first passage. These findings are in accord with Taylor's observations.[3, 4] However, Fukumi and his associates[15] report the isolation of three strains of Type C influenza in Japan by mouse passage technique alone.

Six rhesus *monkeys,* previously used for work in poliomyelitis, were divided into 3 groups of 2 animals each. The first group received 2 ml. of normal amniotic fluid intranasally, the second received 2 ml. of JJ infected amniotic fluid containing 10^8 egg infectious doses of virus intranasally, and the last group the same amount of material subcutaneously. The animals were observed and temperatures recorded twice daily for 17 days following the inoculations. No abnormal changes in the appearance, appetite, or temperature of any of the animals were seen. Antibody developed to the same level in monkeys inoculated subcutaneously or intranasally. However, the levels achieved were much lower than those obtained in mice or ferrets.

TABLE V. RESULTS OF CHALLENGE OF JJ VACCINATED MICE WITH OTHER INFLUENZA VIRUSES

CHALLENGE VIRUS	LD_{50} OF CONTROL MICE	LD_{50} OF JJ VAC. MICE
PR8 Type A	$10^{-6.8}$	$10^{-6.0}$
FM1 Type A′	$10^{-6.0}$	$10^{-5.9}$
Lee Type B	$10^{-4.0}$	$10^{-4.1}$

Cross-Immunity Tests.—A group of 82 young mice was vaccinated with the JJ strain by administering two intraperitoneal inoculations at one-week intervals of 0.5 ml. of virus suspension containing 10^8 egg infectious doses. One week following the last intraperitoneal injection, groups of six mice each were challenged by intranasal inoculation of mouse-adapted strains of PR8, FM1, and Lee influenza viruses in tenfold dilutions. Control mice of the same age were challenged with the same dilutions of virus (Table V). Apparently vaccination with the JJ strain will not inhibit the growth of other types of influenza virus in mice. At the time of challenge ten vaccinated mice and ten control mice were bled in order to study antibody response. The hemagglutination-inhibition titer obtained with the serum from the vaccinated mice when tested with the homologous virus was 1:2048, while no antibodies to the heterologous viruses were demonstrable. The control serum was negative with all strains.

Serological Studies With Human Sera.—In order to make the earlier report[1] more complete, the following data are presented. The sera that had been obtained from 68 children in a children's home which was under study from August, 1946, through May, 1947, showed by means of the HI (hemagglutination-inhibition) test that an epidemic of Type C influenza appeared concurrently with one of Type A-prime. In Fig. 1 the open circles represent

the HI titers to the JJ strain of the pre- and postepidemic pairs of sera obtained from the children, while the solid circles represent the titers of pairs of acute and convalescent sera from adults in the children's home who had illnesses during the epidemic period. It may be seen that 52 of the 68 children show an increase in antibodies to the JJ strain and that in all but 3 instances the pre-epidemic titer was less than 32, while, of the 16 who registered no response, all but 3 had initial titers ranging from 64 to 512. On the other hand, the initial titers of the 15 adults were considerably higher than those of the children, and only 4 of them showed a rise in titer in the convalescent state.

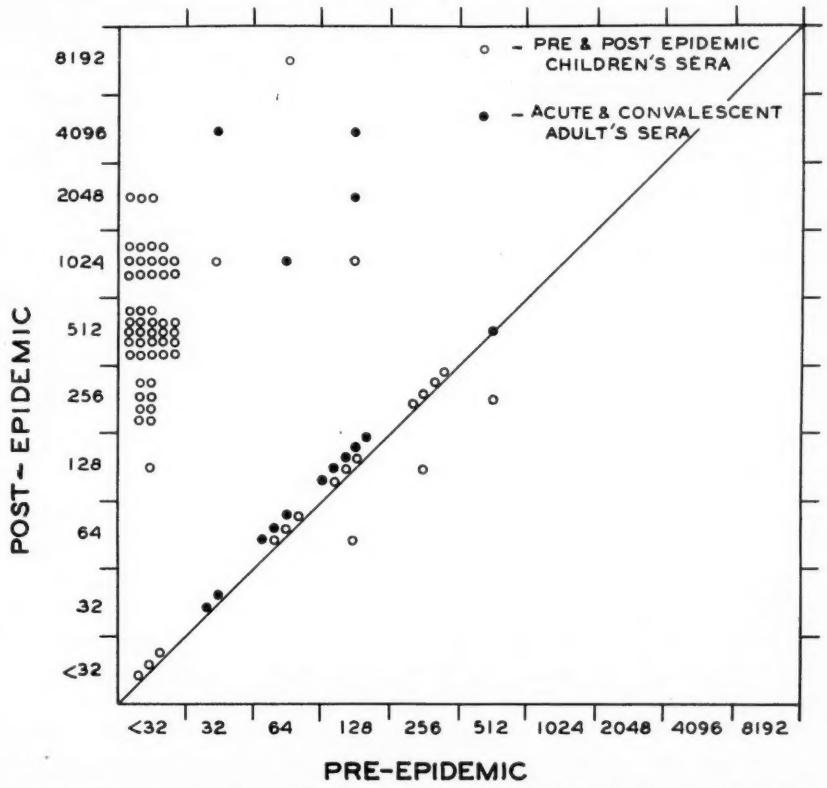

Fig. 1.—Hemagglutination-inhibiting antibodies against the JJ (Type C) influenza virus.

In order to confirm the serologic patterns found in the HI tests, a few cases were selected for further study by complement-fixation and in ovo neutralization reactions. The results of the three types of tests are recorded in Table VI. Case 1 is the patient from whom the JJ strain of virus was isolated. All the others are the children or adults in the children's home. The first group of three cases was chosen because it demonstrated antibody increase to the JJ strain, while the A, A-prime HI titers either remained the same or, as in Case 8, actually decreased. The second group of three, on the other hand, experienced rises in titer to the A, A-prime group of viruses and had constant

high levels of antibody to the JJ strain. Cases 57 and 58 were interesting from the point of view that they were brother and sister showing the same serologic pattern. Both demonstrated decrease in antibody to Type A, increase in antibody to Type A-prime, and had a constant high level of antibody to Type C. The last three cases demonstrate dual infection with rises in titer to Types A, A-prime, and C influenza viruses. In all cases the CF or EN tests confirm the HI values and give further proof that the JJ strain of virus is immunologically distinct.

TABLE VI. COMPARISON OF ANTIBODY RESPONSES AS MEASURED BY HEMAGGLUTINATION-INHIBITION (HI), COMPLEMENT FIXATION (CF), AND OVO NEUTRALIZATION (EN) TESTS

SERUM (NO.)	STAGE	TYPE A			TYPE A′			TYPE B		TYPE C		
		PR8*			RHODES*	FM1*	RHODES*	LEE*		JJ*		
		HI	CF	EN	HI	CF	EN	HI	CF	HI	CF	EN
2†	Acute	32	<8	10	<32	<8	16	512	<8	32	<8	6
	Convalescent	32	<8	8	<32	<8	10	512	<8	4096	128	646
3†	Acute	64	<8	<4	32	<8	40	128	<8	128	<8	128
	Convalescent	64	<8	<4	32	<8	40	128	<8	4096	64	4096
8	Pre-epidemic	64	16		1024	<8	102			<32	8	16
	Postepidemic	32	<8		128	<8	25			512	16	1024
17	Acute	<32	<8	10	<32	<8	4	32	<8	128	<8	405
	Convalescent	512	64	644	64	32	128	32	<8	128	<8	256
25	Prevaccination									256		161
	Acute	1024	8	64	32	<8	4	64		256	<8	128
	Convalescent	2048	16	512	128	<8	8	64		256	<8	128
28	Prevaccination	32								512		512
	Pre-epidemic	128	<8	25	32	<8	<4			512	<8	512
	Postepidemic	512	8	160	512	16	32			256	<8	415
57	Pre-epidemic	2048	16	512	64	<8	4			256	2	109
	Postepidemic	256	8	128	1024	64	512			128	2	128
58	Prevaccination									256		512
	Pre-epidemic	1024		512	64		24			256		512
	Postepidemic	256		32	256		128			256		128
1†	Acute	64	<8	8	<32	<8	8		<8	64	<8	64
	Convalescent	128	<8	20	32	<8	32		<8	2048	64	646
4	Acute	512	<8	40	<32	<8	<4	64	<8	<32	<8	<4
	Convalescent	4096	64	512	2048	32	1024	64	<8	512	64	16
20	Pre-epidemic	128			<32		<32			128		160
	Postepidemic	512			256		202			1024		>4096

*Strains of virus against which sera were tested.
†Adults.

In a search for further evidence of the occurrence of influenza C, available pairs of acute and convalescent sera from previously identified epidemics of either Type A or Type B were examined by the HI test. These sera covered a span of years from 1936 through the spring epidemic of 1952. Whereas, antibodies to the JJ strain were present in each group, no evidence of acute illness, as shown by increase in antibody level, was found until the original case (JJ) in 1950. In studying the Type A-prime influenza epidemic of 1951 two cases were observed with antibody increases in the convalescent sera to both Type A-prime and Type C influenza. In one case both viruses were isolated simultaneously from the throat washing.[13] While studying the Type B epidemic of 1952 at an institution for children and young adults whose age span was from 7 to 25 years, two cases of Type C influenza occurred during the last week of the Type B epidemic.[16] In comparing bleedings from 22 persons

in this institution taken three months before and two months after the Type B epidemic, 4 additional Type C infections were encountered, 2 of them having previously been infected with Type B. However, there was no indication of simultaneous infection. Studies at the Great Lakes Naval Training Station identified a series of cases during the same season.[17] Recently one adult has been seen who had an afebrile respiratory illness followed about one week later by a febrile episode; they were shown serologically to have been influenza C and A, respectively.

Human subjects vaccinated with the FM1 strain of Type A-prime influenza with a polyvalent Type A vaccine containing PR8, FM1, and Cuppett strains and with a Type B (Lee strain) vaccine showed no change in the Type C antibody titers while responses to the specific strains were evident. Nevertheless, in all these tests the number of individuals with high levels of antibody against the JJ strain was large enough to emphasize the fact that infection with this agent has been widespread at least since 1936.

HI tests with pairs of acute and convalescent sera from groups of cases of rubeola, rubella, infectious mononucleosis, atypical pneumonia, the common cold, and unidentified acute respiratory diseases all gave negative results.[1] Rabbit antimumps serum, mouse sera from animals immunized against Melnick's Texas, Ohio, and Connecticut strains of Coxsackie virus[18] and mouse anti-PVM serum had no HI antibodies against either JJ or 1233 strains. Sera from human beings and ferrets with high levels of antibody against the JJ virus showed no HI antibody when tested against a strain of Newcastle disease virus (NDV).

COMMENT

The results of these studies emphasize the fact that an apparently stray virus was converted by investigation of the epidemiological connotation of serologic results into the etiologic agent of an extremely widespread infection. Since neutralization of the virus was so commonly exhibited to high titer with the sera of adults, it became of interest to determine the occurrence of that effect in younger individuals in order to ascertain the significance of this neutralizing activity. In the course of the effort it was noted that many children had low titers or no demonstrable antibody to the virus and an epidemic of the disease was demonstrated in children serologically. Clinically it is not yet well defined except as it resembles mild influenza. Continued search has yielded only a few additional cases, but the ubiquity of antibodies indicates that this infection must be primarily acquired very early in life as a mild undifferentiated respiratory illness of young children or as a common subclinical infection. The few adults we have observed at first hand during the natural infection have had distinct signs and symptoms referable to the upper respiratory tract with little or no fever. On the basis of present knowledge the disease appears to occupy an intermediate position between the more characteristic influenzas and typical common colds. The fact that the virus appears to limit its site of infection to the turbinates of experimental animals is of interest in this respect. The general features, the evidence of its distribu-

tion, and the properties of the virus seem to justify the designation influenza C, if only to stimulate further investigation of its significance in the field of respiratory disease.

SUMMARY

The method of isolation and maintenance of Type C influenza, its hemagglutinating characteristics with red cells from various species, the adsorption and elution from chicken erythrocytes, adaptation to animals, and serologic studies are presented.

The significance of the virus as a causative agent of human disease is discussed in relation to epidemiological interpretations derived from serologic investigations in population groups.

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TYPE C INFLUENZA VIRUS

II. Intranasal Inoculation of Human Individuals

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THE PRECEDING papers have defined the independent character of the Type C influenza virus from other types of commonly recognized hemagglutinating respiratory viruses.[1, 2, 3] Despite the fact that serologic evidence was presented to show the extent to which infection with Type C virus had occurred in a children's home, the simultaneous occurrence of the A-prime epidemic made it difficult, in reviewing the clinical records, to draw conclusions as to destructive clinical manifestations associated with Type C infection. Therefore, it was necessary to obtain more evidence of the clinical manifestations in man. With this latter thought in mind, the present studies were undertaken.

MATERIALS AND METHODS

Eleven women, who were patients in a State Hospital,* were chosen for the study. They were fairly well adjusted to their environment and all of them had some daily occupation in the hospital. Ten days prior to the inoculations, all of the women were examined carefully and had chest x-rays, blood and urine analyses. In no instances were any sharp abnormalities detected. At this time blood specimens were also obtained.

The group was then divided on the basis of their hemagglutination-inhibiting antibody titers against the JJ virus. Six with the lowest titers were sprayed intranasally with 1 ml. of amniotic fluid containing $10^{9.5}$ EID_{50} of the JJ strain of Type C influenza virus which had been carried for 12 passages in the amniotic sacs of eggs. This was administered by one of the investigators and was done in a small utility room, using a hand atomizer delivering approximately 0.5 ml. in each nostril. During the course of the spraying there was a considerable amount of coughing and sneezing in the face of the sprayer.

The remaining 5 women were sprayed with normal amniotic fluid in the same fashion in another room. Following these procedures, the two groups were kept apart for four hours. They were then placed together, in a large suite with an open bed dormitory, for the next two weeks. During this latter interval, they were in contact only with necessary hospital personnel. For both groups, rectal temperature, pulse, and respirations were recorded twice daily for four days prior to the spraying and for sixteen days afterward. Daily visits and examinations were made during the first week, and again on the tenth and fourteenth days after the inoculations. Throat garglings with beef broth were obtained on the first four days and again on the seventh day. The throat garglings were obtained in the morning and inoculated into eggs the same afternoon.

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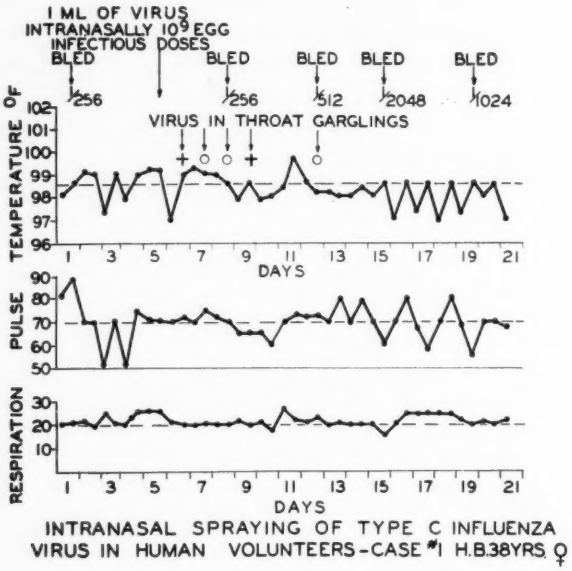
Blood specimens were obtained on the third, seventh, tenth, and fourteenth days after the spraying was done. The individual sera were placed in rubber-stoppered tubes, stored at 6° C., and, after all the specimens had been collected, examined in hemagglutination-inhibition tests, at the same time.

Virus Isolations.—The throat washings were inoculated into the amnion of 10-day-old chick embryos.[3]

Hemagglutination-Inhibition Titrations.—The methods used were the same as those previously described.[3]



INTRANASAL SPRAYING OF TYPE C INFLUENZA VIRUS IN HUMAN VOLUNTEERS - CASE #1 H.B. 38 YRS ♀

RESULTS

Following administration of the sprayed material no sharp changes were noted in the condition of the subjects nor were there any significant complaints. One of the women who received the virus complained of a stuffy nose and received codeine for a headache. This occurred on the day after she received the spray. At this time no abnormalities could be seen on examination of the nose and throat nor were there any further complaints on succeeding days. One of the subjects receiving the normal amniotic fluid complained of chilliness and some headache for two days following administration of the fluid. In no instance was a change noted in the temperature, pulse, or respirations of any of the subjects.

Fig. 1 presents the observations made on one of the individuals who received the virus. It shows that the temperature, pulse, and respirations remained within normal limits. Also shown are the times when the virus was given and when blood and throat specimens were obtained. The titers of hemagglutination-inhibiting antibody against the JJ virus are shown as are the results of attempts to isolate virus from throat washings. The virus was

isolated on two occasions, one day and four days after the spray was administered. The sharp rise in antibody titer reached a peak on the tenth day after administration of virus.

The results of attempts to isolate virus from all of the subjects are presented in Table I. A positive sign indicates that virus was recovered in eggs from a particular sample.

TABLE I. VIRUS RECOVERED FROM THROAT GARGLINGS OF INDIVIDUALS SPRAYED WITH THE JJ STRAIN OF TYPE C INFLUENZA VIRUS

	SPRAY	DAYS AFTER SPRAYING					CHANGE IN HI TITER
		1	2	3	4	7	
L. T.	1 ml. I.N. JJ Virus 10^9 EID_{50}	+	ct*(0)	+	+	0	128/8192
H. B.	1 ml. I.N. JJ Virus 10^9 EID_{50}	+	ct(0)	0	+	0	256/1024
J. M.	1 ml. I.N. JJ Virus 10^9 EID_{50}	+	ct(0)	+	0	0	64/512
M. B.	1 ml. I.N. JJ Virus 10^9 EID_{50}	0	ct(0)	+	0	0	256/1024
M. L.	1 ml. I.N. JJ Virus 10^9 EID_{50}	0	+(0)	+	0	0	256/256
F. L.	1 ml. I.N. JJ Virus 10^9 EID_{50}	0	ct(0)	0	0	0	128/128
5 controls	1 ml. I.N. normal amniotic fluid	0	ct(0)	0	0	0	No significant changes

*All eggs contaminated on first isolation attempt. Reinoculated after CO_2 freezing. Parentheses indicate results of reinoculation.

On at least one occasion, 5 of 6 who were subjected to spraying of virus had virus present in their throat garglings. Twenty-four hours after receiving the virus, 3 individuals were positive. Unfortunately, the data on the second-day samples cannot be fully evaluated because of contamination. On the third day the largest number of positive tests was obtained; on the fourth day only 2 had virus present while after seven days none were positive. Also summarized in the table are the results of attempted isolations of virus in the control group, all of which were negative. The last column of the table shows the preinfection hemagglutination-inhibition titers as the numerator and the titers obtained fourteen days after the spraying as the denominator. It is of some interest to note that the highest titer was observed in the individual from whom virus was isolated on three occasions and that this person was the youngest of the group (15 years). All of the virus isolations were checked with specific antisera and were shown to be inhibited by anti-JJ sera.

Table II presents in more detail the development of the hemagglutination-inhibiting antibody in the infected group. The levels of antibody shown for the preinfection samples, obtained 10 days before the virus was given, were the same as those obtained 3 days after spraying with the exception of subject 6 where an insignificant drop was noted. One week after infection, 2 subjects already showed some evidence of antibody increase and by 10 days the maximum levels of antibody were achieved. No change in the antibody titer

of the fifth individual occurred despite the fact that virus was recovered twice. In the last one of the infected group, no antibody developed nor was virus found on any occasion. The controls showed no significant changes. All sera were tested and showed no changes in antibody against strains of virus of the A, A prime, or B types.

TABLE II. HI ANTIBODY RESPONSE OF ADULT WOMEN TO INTRANASALLY SPRAYED JJ STRAIN OF TYPE C INFLUENZA VIRUS

	DAYS FROM SPRAYING					VIRUS PRESENT
	−10	+3	+7	+10	+14	
1	128	128	1,024	16,384+	8,192	3/5
2	256	256	512	2,048	1,024	2/5
3	64	64	64	512	512	2/5
4	256	256	256	1,024	1,024	1/5
5	256	256	256	256	256	2/5
6	128	64	128	128	128	0/5
5 controls	No significant antibody changes					0/5

Although interesting data were thus obtained concerning subclinical infection, no added knowledge of clinical manifestations of the disease was obtained from the subjects under study. However, seven days after the spraying, the investigator, who had sprayed the active virus, first noted a slight postnasal irritation with some mucous discharge. The following evening the nasal irritation and mucoid discharge were more pronounced and a slight cough developed. On the third day, malaise and generalized aching involving the eyes were observed together with a moderate nasal discharge and some sneezing. On this day, throat washings and a blood specimen were obtained; Type C influenza virus was isolated from the washings. No fever or chilliness was noted but that evening the generalized aching sensation persisted to the point where aspirin was taken. These symptoms continued for the next two days. No other complaints were noted but a feeling of lack of energy persisted for a considerable period of time. Even in this instance, it is doubtful whether the clinical manifestations can be considered characteristic.

This case was a rather mild illness with no clearly defined signs yet nine days after the spray procedure virus was isolated. During the period between the spray procedure and the time of illness, no contact with the JJ virus occurred and the degree of exposure during the spraying was so great that it seems most likely that infection occurred then. If this is correct the incubation period was seven days, considerably more than observed after induced infection with Type A or B viruses.[4, 5] The virus was shown to be identical with the JJ strain and antibodies developed in the late serum samples, rising from a level of 1/512 on the second day of illness to 1/2,048 on the twelfth day and 1/1,024 on the fourteenth day.

SUMMARY

Six women who each received by intranasal spray 1 ml. of amniotic fluid containing the JJ virus did not develop clinical evidence of illness during a

sixteen-day observation period. However, virus was recovered from throat garglings of 5 of the 6, and 4 of the 6 developed significant rises in anti-JJ antibodies in the late serum samples.

The investigator who sprayed the virus developed a mild type of acute respiratory disease which had its onset seven days after the spray procedure was carried out. A virus was isolated from the throat garglings obtained on the second day of illness and was found to be identical to the JJ strain of virus. A significant antibody rise against the JJ strain also developed in the convalescent serum sample.

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ACTH THERAPY IN ACUTE VIRAL HEPATITIS

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ATTEMPTS to shorten the course of convalescence of viral hepatitis by means of dietary adjuvants,[1] antibiotics and other supplementary medications[2-4] are recorded in recent medical literature. Most of these have resulted in but equivocal to slight success. Even with the enforced use of rest and a nutritious diet, the course of the disease has not been materially altered.[5] The present report deals with experience using ACTH in viral hepatitis.

Although not clearly established as yet, there seems little doubt that differences in virus type and individual host constitutional differences account for the variations often seen in the severity and duration of viral hepatitis. Because of these uncontrollable factors evaluation of therapy is exceedingly difficult, especially if the differences between the treated and control groups are small. One would anticipate that such factors might be held to a minimum if one were given the opportunity to study a single epidemic involving a narrow age group. Such an opportunity was not available for the present study.

MATERIAL AND METHODS

Investigation of the possible value of ACTH in acute viral hepatitis was begun in 1949 when this material first was available for use in patients with hepatic disease. The total experience covers a four-year period during which there was a very slow accumulation of cases and a variety of dosage schedules used. For the most part the patients used in this study were young soldiers 18 to 23 years of age. Those soldiers developed hepatitis while stationed in near-by areas or while on a patient status in this hospital for other causes. Many of these patients were believed to have the "long-incubation-period" hepatitis since they had received plasma or blood for their underlying medical or surgical problems and developed hepatitis after the passage of 60 to 120 days.

The patients were selected so that the therapeutic study was begun within the first seven days of jaundice. Alternate patients were used as controls, with the same criteria of selection for these as for the treated patients. Twelve were treated before the program for selection of controls was initiated; this accounts for the difference in numbers of treated and control cases. All patients used were managed with a basal regimen consisting of bed rest with bathroom privileges and an ad lib intake of a diet high in protein and calories. Sodium chloride was restricted, and in all cases the diet was supplemented with 2 Gm. of potassium chloride daily. In the later stages of this study when intravenous infusions of ACTH were used, the control patients were also given a slow intravenous infusion of 5 per cent glucose to which was added a placebo which had the physical appearance of ACTH.

When the total serum bilirubin reached 1 mg. per cent or less and the Bromsulphalein retention was less than 10 per cent, the patients were allowed to become progressively active

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over a three-week period. If no signs or symptoms of recurrent hepatitis appeared in this period, they were permitted to leave the hospital but were returned in thirty days for a final examination. Needle biopsy of the liver was taken in all patients at the time the diagnosis was made and at varying periods thereafter. It was possible to compare the histologic appearance of the liver in groups of treated and control patients at ten, twenty, and thirty days after the initial biopsy.

The first schedule of ACTH consisted of a single injection daily of 100 mg. for two days, then 75 mg. a day for seven days, all by the hypodermic route. The first fourteen patients were treated in this way. As experience with this medication was accumulated, other schedules were used. The second regimen consisted of 20 mg. ACTH dissolved in 500 ml. of 5 per cent glucose given intravenously by slow drip (eight to twelve hours) daily for ten days and then abruptly stopped. While there seemed to be a prompt response to this treatment, the rapid improvement ceased when the ACTH was stopped. Of five patients receiving this course of therapy, there was one relapse when the medication was discontinued. The next ten patients were treated with a slow intravenous drip of ACTH given for two to five days, followed by intramuscular ACTH in divided doses until the total serum bilirubin reached 1 mg. per cent. In the hope that sustained stimulus of the adrenal cortex would further shorten the course, the next nine treated cases were given ACTH by slow intravenous drip daily for the entire period of time that the total serum bilirubin remained above normal. This schedule was tapered so that 10 mg. ACTH were administered for the first five days, then 5 mg. ACTH for the second five days, and 2.5 mg. ACTH were administered daily until the bilirubin reached normal. The final, and thus far the most successful, course of therapy has consisted of 20 mg. of intravenous ACTH given daily for five days followed by 10 mg. for five days, and then 5 mg. daily until the serum bilirubin was normal. To date eleven patients have been treated with this latter course.

Because of the variety of therapeutic regimens attempted, the control patients were divided into two groups: those who received 5 per cent glucose in water plus a placebo intravenously and those who did not.

Also included in this report is an additional group of six cases of so-called "cholangiolitic hepatitis."[6] Since no comparable controls were available for these patients, their progress before and during ACTH therapy was compared to evaluate the effect of the medication. This group presented a rather uniform picture with jaundice present for periods of time varying from days to months. Also present were pruritus, a moderately high alkaline phosphatase, and an elevated total cholesterol. Some of these patients showed normal flocculation and turbidity tests. Liver biopsies were taken in five of the six and showed the usual evidences for viral hepatitis plus a prominence of bile thrombi.

RESULTS

A total of forty-nine treated and thirty-six control cases were used in this study. The scattergrams (Figs. 1-4) and Table I show the general patterns of response of the liver function tests to the different schedules of ACTH. The mean duration of abnormal tests as shown in the scattergrams tended to be shorter as greater and more sustained adrenal stimulus was achieved. This was particularly noticeable in the charts showing the effect of ACTH upon the duration of abnormal serum bilirubin and urine bilirubin tests. Although the mean values for the treated and the control groups are different, these differences are not statistically significant.

The six cases of "cholangiolitic hepatitis," on the other hand, seem to show a clear relationship between the time that therapy was started and improvement began. The course of disease in three of these is illustrated.

TABLE I. MEAN DURATION IN DAYS OF POSITIVE LIVER FUNCTION TESTS AND MEAN MAXIMUM BILIRUBIN PEAK COMPARED IN CONTROLS AND GROUPS ON VARIOUS DOSAGE SCHEDULES OF ACTH

	I CONTROL NO I-V GLUCOSE	II CONTROL I-V GLUCOSE	III ACTH SINGLE DOSE SUBCU. 10 DAYS	IV ACTH I-V FOR 10 DAYS	V ACTH I-V 2-5 DAYS THEN I-M UNTIL SB NORMAL	VI ACTH I-V DAILY UNTIL SB NORMAL 10-5-2.5	VII ACTH I-V DAILY UNTIL SB NORMAL 20-10-5
Serum Bilirubin	45.3	33.6	38.7	35.4	27.7	26.3	23.2
Urine Bilirubin	19.2	12.2	12.4	9.7	9.7	9.8	6.3
Thymol Turbidity	44.2	36.1	24.9	20.7	22.2	13.6	15.6
Cephalin Flocculation	25.8	20.2	14.9	11.3	16.1	12.7	21.1
Maximum Bilirubin	12.8	7.6	11.9	8.4	7.7	10.2	7.3

The first patient (Fig. 5) was a 30-year-old officer who was treated initially with intravenous glucose. His hepatitis seemed to respond to the therapy but after a period of thirty-five days and for no obvious cause his disease worsened. By six days later the bilirubin value was again at its previous peak. Along with this there was seen a recurrence of the patient's symptoms with anorexia, fetor hepaticus, pruritus, general malaise and lethargy. At this point it was decided to discontinue the glucose and start intravenous ACTH. The response of the patient to the new medication was dramatic. There was a return of well-being, the appetite improved, and the liver function tests rapidly returned to normal. An uneventful recovery ensued. A follow-up examination six months later showed no evidence of residua of hepatic disease.

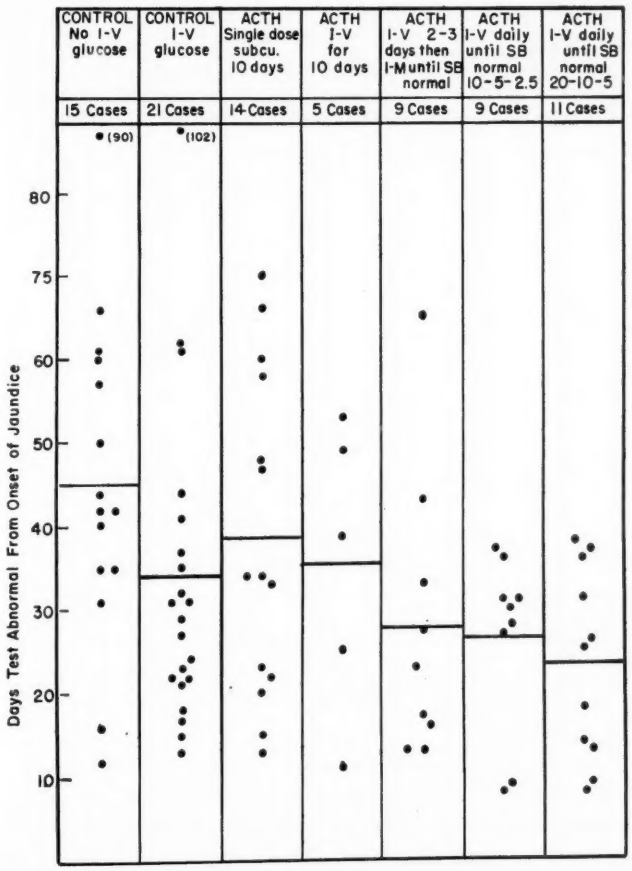

Fig. 1.—ACTH therapy in acute viral hepatitis. Number of days from onset required for serum bilirubin to return to normal compared in controls and on various dosage schedules.

The second example (Fig. 6) was a 29-year-old soldier who developed jaundice and was admitted to another hospital. He retained deep jaundice and pruritus until the time of his transfer approximately 170 days later. He had been treated with restricted activity and a high caloric diet throughout the entire period with no change in his course. The physical examination revealed a deeply jaundiced patient whose liver was palpable 6 cm. below the right costal margin. The initial total serum bilirubin value was 9.6 mg. and remained in this range until the time intravenous ACTH therapy was started. A sharp drop in the serum bilirubin followed shortly thereafter, which was paralleled by the other liver

function tests. There was an associated return of well-being and appetite. When the serum bilirubin reached normal, the ACTH was discontinued. Follow-up examination six months later showed no evidence of hepatic disease.

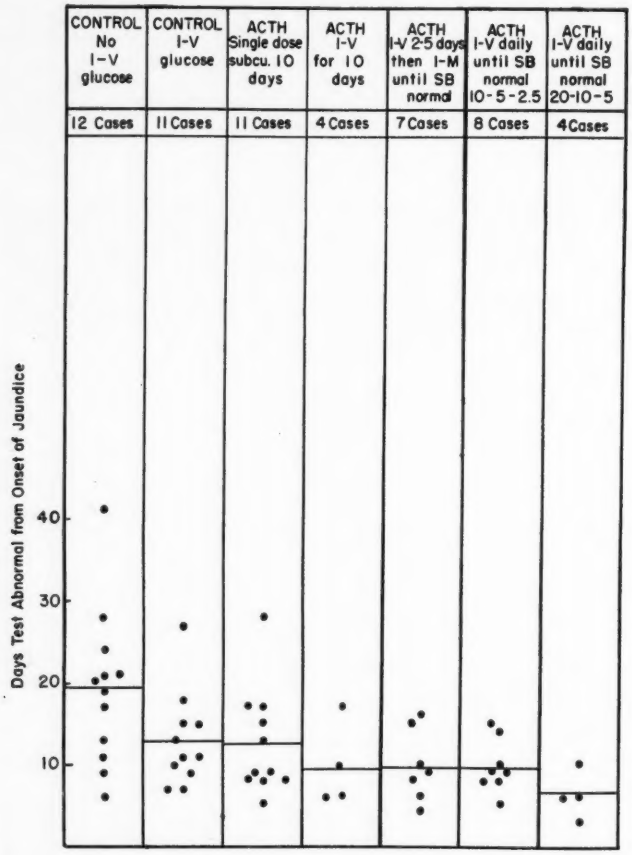

Fig. 2.—ACTH therapy in acute viral hepatitis. Number of days from onset required for urine bilirubin to return to normal compared in controls and on various dosage schedules.

The third patient (Fig. 7), a 32-year-old officer, was seen six days after he first developed jaundice. At that time he showed marked icterus, generalized pruritus, and an enlarged tender liver. He was complaining of anorexia, fatigue, and right upper quadrant tenderness aggravated by activity. The early hospital course was characterized by a rising serum bilirubin and an increasing pruritus. Intravenous ACTH therapy was begun fifteen days after the onset of jaundice. There was noted an immediate and dramatic response with a return toward normal of the liver function tests, a disappearance of pruritus, and improvement in well-being and appetite. After forty-five days of intravenous ACTH, the serum bilirubin reached normal and the medication was stopped. Thereafter, the patient's course of recovery was uneventful.

COMMENT

As experience with the use of ACTH in acute viral hepatitis gradually accumulated, it became clear that a larger and more sustained stimulus of the adrenal was necessary to shorten the course of positive liver function tests. The

tendency for the mean values of duration of positive liver function tests to be lowered as the adrenal stimulus was increased reflects the success of this therapy. It is to be emphasized, however, that because of the wide individual variation in duration of abnormal liver tests observed in these small groups, none of the differences between the means of the treated and control groups were found to be statistically significant. Since the mean bilirubin peaks for some

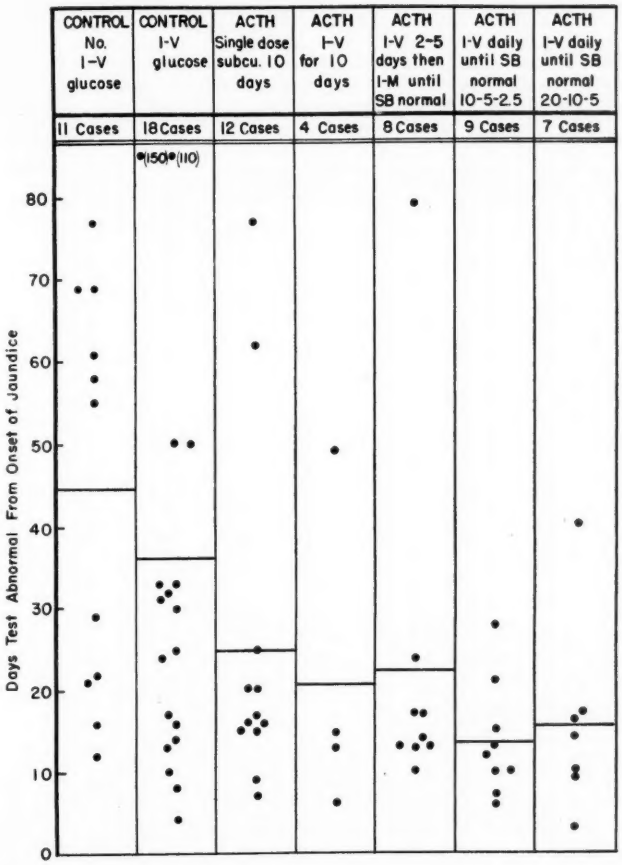

Fig. 3.—ACTH therapy in acute viral hepatitis. Number of days from onset required for thymol turbidity to return to normal compared in controls and on various dosage schedules.

of the treated and control groups are different, it is likewise doubtful that they can validly be compared. It has previously been shown by Zimmerman and associates[7] and by others[8] that the duration of jaundice is generally proportional to the peak of bilirubin reached, that is, the higher the peak of icterus the longer is required for the bilirubin to return to a normal value. Because of this the only groups in the present series which are comparable in any way are groups II, V, and VII (Table I).

In all cases of viral hepatitis where ACTH was used intravenously there occurred a sharp drop in bilirubin within forty-eight hours (this did not occur

in one patient with obstructive jaundice where ACTH was used inadvertently). It is possible that the effect of ACTH upon the liver function tests studied is a nonspecific suppression and under these circumstances they cannot be used to follow the progress of the patient. It was hoped that the liver biopsies would be of decisive value in this regard. When comparing the biopsies, taken at various intervals in the control and treated cases, however, the differences noted were not sufficiently clear-cut to be quantitated even on a rough scale.

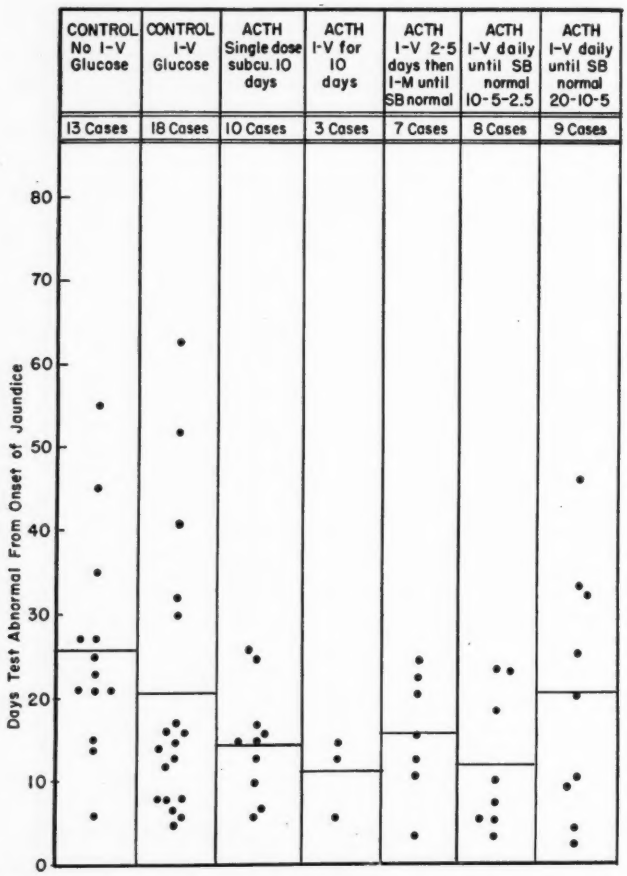

Fig. 4.—ACTH therapy in acute viral hepatitis. Number of days from onset required for cephalin flocculation to return to normal compared in controls and on various dosage schedules.

Despite the possibility that ACTH influenced the liver function tests in a nonspecific way, they were used as a basis of therapeutic response just as they are with the ordinary case of viral hepatitis. The patients in all groups received a minimum of one month of bed rest followed by progressive ambulation when the serum bilirubin reached normal (1 mg. per cent). In one patient in a treated group (groupV, Table I) and in one patient in a control group (group II, Table I) there occurred a relapse after ambulation was begun.

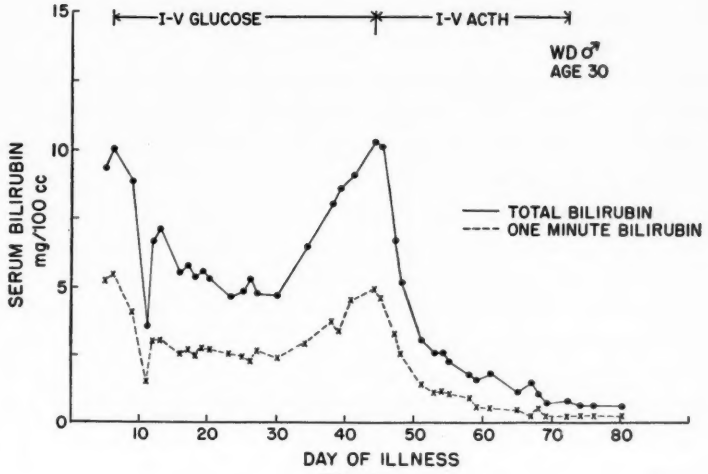

Fig. 5.

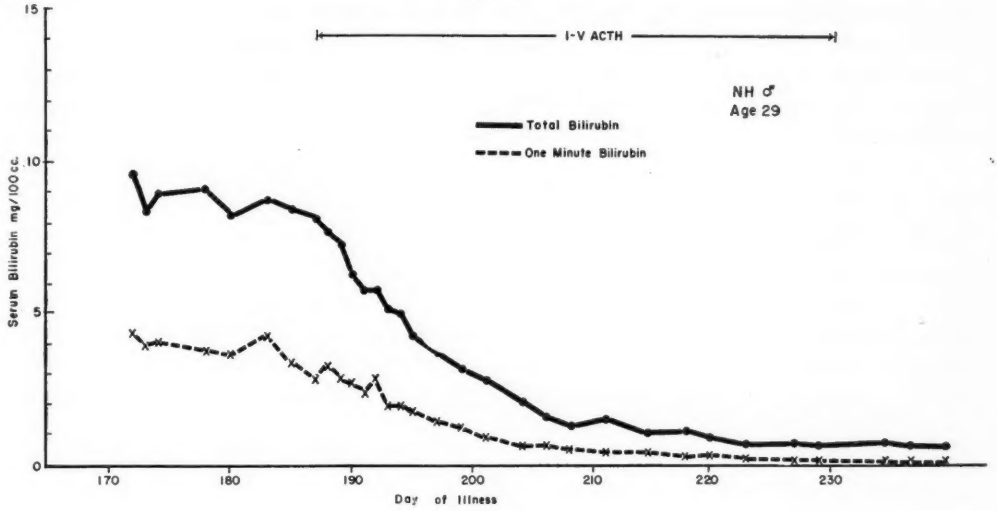

Fig. 6.

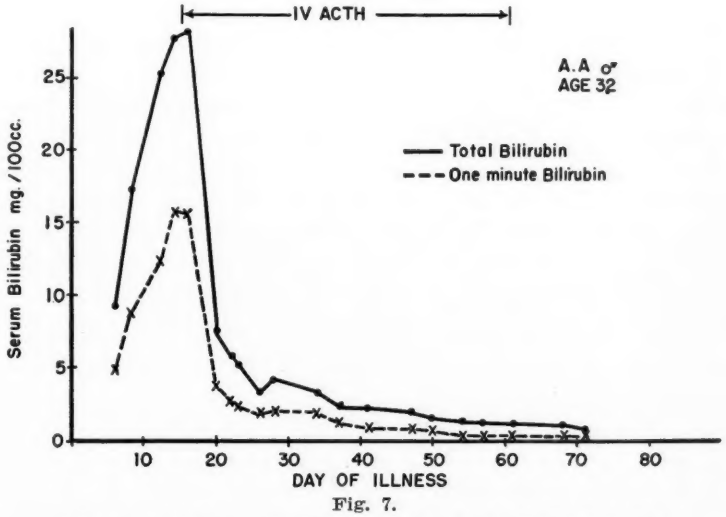

Fig. 7.

Figs. 5, 6, and 7.—ACTH therapy in cholangiolitic hepatitis.

Although the differences in the various treated and control groups might have occurred by chance alone, simple inspection of the scattergrams seems to indicate a definite shortening of the duration of abnormal liver function tests.

The response of the hepatic tests in the patients with cholangiolitic hepatitis seems more clear-cut and striking. In all of these cases the serum bilirubin was either rising or had remained stationary for a definite period of time prior to therapy. With the administration of ACTH there occurred a prompt and decisive improvement. All of the patients in this latter group (up to six months after onset of jaundice) recovered uneventfully.

Certain side effects were noted in the patients who received the intravenous ACTH. These included rapid weight gain, acne, moonface, glycosuria, and small rises in blood pressure. Upon withdrawal of therapy, joint pains in the shoulders, knees, and ankles were occasionally noted. None of these patients developed visible edema or ascites. ACTH was discontinued after thirty days in one patient (group VI, Table I) when there occurred a marked exacerbation of infection of a pilonidal cyst. This responded promptly to antibiotics and ACTH withdrawal. In no instance was there noted a worsening of hepatitis while receiving ACTH.

Previous experience with ACTH in acute viral hepatitis is scanty. Thorn and his co-workers[9] published one case of aplastic anemia complicated by hepatitis which seemed to respond to ACTH. Colbert and associates[10] reported treatment with intramuscular ACTH in five patients for periods up to eight days. Two of these patients developed a recurrence of jaundice when ACTH was discontinued. A sharp drop in the serum bilirubin and a less clear response in the thymol turbidity and cephalin cholesterol flocculation tests were noted. It was the belief of the authors that ACTH showed beneficial effects in this disease.

Since then Rifkin and his group[11] have used ACTH in three patients and cortisone in one patient with acute hepatitis. All showed rapid improvement. Rifkin did not believe, however, that the basic disease was altered with this therapy.

From the present study it would be most difficult to form a definite conclusion. A comparison of the mean duration of abnormal serum bilirubin, urine bilirubin, and thymol turbidity in treated and control groups leads one to believe that when used early ACTH may be effective in shortening the course of hepatitis in some and possibly in preventing the occurrence of late cases in others. Since the difference of these means were not found to be statistically significant, the accumulation of a larger number of cases must be awaited before definite conclusions can be drawn. There was clearly no worsening of the disease by ACTH in the treated cases. The use of ACTH in six cases of "cholangiolitic hepatitis" where there was deep jaundice for as long as six months has been successful in terminating the biochemical manifestations, with resulting complete recovery.

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STEROID EXCRETION PATTERNS IN ACUTE VIRAL HEPATITIS, WITH AND WITHOUT ADRENOCORTICOTROPHIN INFUSION

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THE first published report dealing with 17-ketosteroid excretion in liver disease appeared in 1941.[1] Since then, similar studies have been made, and all have reported significantly decreased levels of urinary 17-ketosteroids.[2-16] Glass and associates[17] and later Zarrow and associates[5] noted low levels of urinary androgens in cirrhosis of the liver. Variable results of urinary 17-ketosteroid levels following the administration of testosterone have been reported.[3, 6, 11, 13, 18] The excretion of increased amounts of corticosteroids in the urine of patients with hepatic cirrhosis has been reported.[8, 10, 11, 14] Data from in vitro studies show that the liver is capable of inactivating the cortical steroids by reduction of the A ring,[19] and that the liver can oxidize certain of the cortical steroids with a 17-hydroxyl group to 17-ketosteroids.[20] Thus, there is much evidence that the liver plays a major role in chemically altering the cortical hormones.

By studying the corticosteroid excretion in patients with various types of liver disease it was hoped that it would be possible to determine if the lowered 17-ketosteroid excretion in liver disease might be the result of, or related to, a decreased production of corticosteroids. Also, by producing an increased level of adrenal cortical steroid by the intravenous administration of ACTH in patients with liver disease (viral hepatitis), and comparing the urinary 17-ketosteroid output to a "control" group of patients with mild chronic hepatitis similarily treated, an attempt has been made to determine if the lowered 17-ketosteroid excretion might be the result of an impaired conversion of corticosteroids to 17-ketosteroids.

CLINICAL MATERIAL AND METHODS

The following cases were studied: (a) acute infectious hepatitis (serum hepatitis and infectious hepatitis), 38 cases; (b) portal cirrhosis, varying degrees of severity, 12 cases; (c) obstructive jaundice, early, 6 cases; (d) chronic hepatitis, 10 cases; (e) normal persons (aged 20 to 40 years), 16 cases.

The diagnosis in all cases of cirrhosis was confirmed by liver biopsy, and in the cases of obstructive jaundice by laparotomy. The diagnosis of chronic hepatitis was based on the following: (1) history of acute hepatitis; (2) characteristic symptomatology or physical findings persisting or recurring six months or longer after a bout of acute hepatitis; (3) minimal or no abnormalities in liver function tests; and (4) liver biopsy abnormalities.[21]

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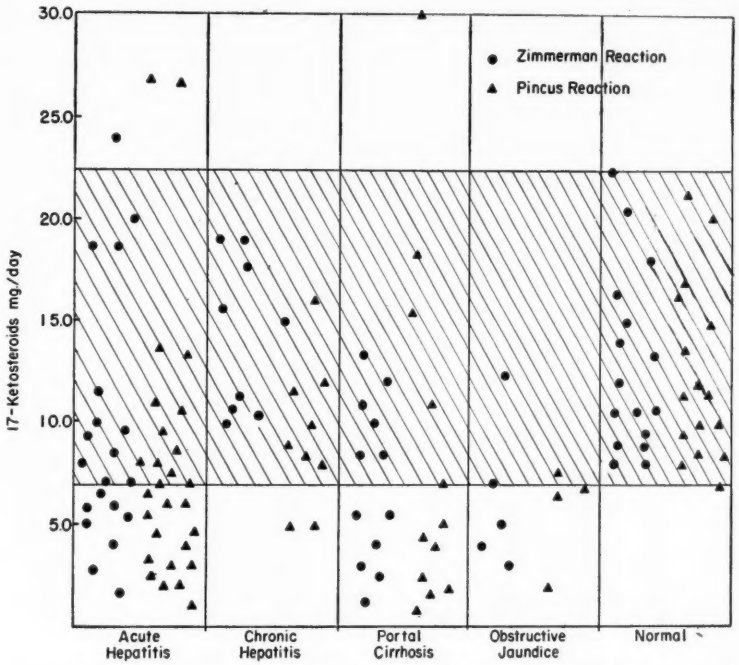

Fig. 1.—Daily 17-ketosteroid levels in normal persons and patients with liver and biliary tract disease. The shaded area represents the range of normal persons.

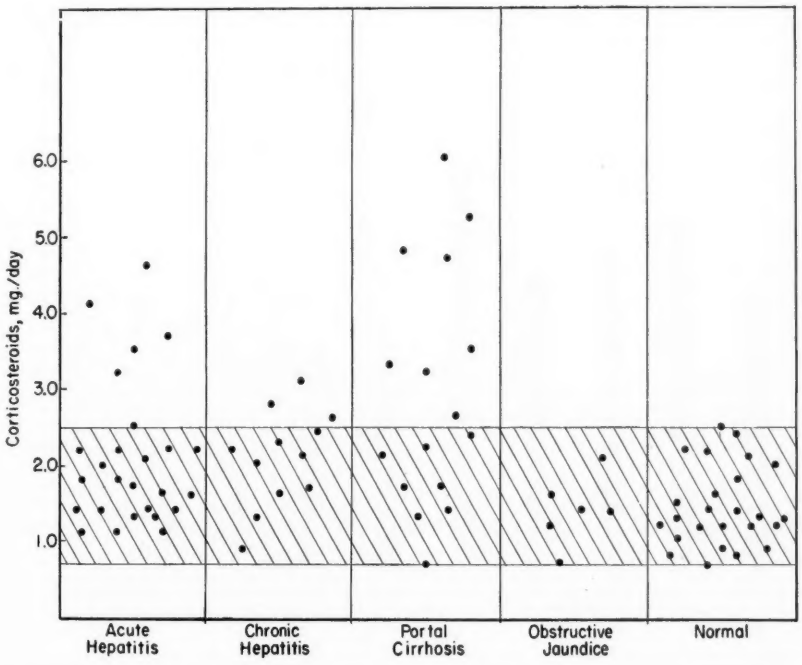

Fig. 2.—Daily urinary formaldehydogenic corticosteroid levels in normal persons and patients with liver and biliary tract disease. The shaded area represents the range of normals.

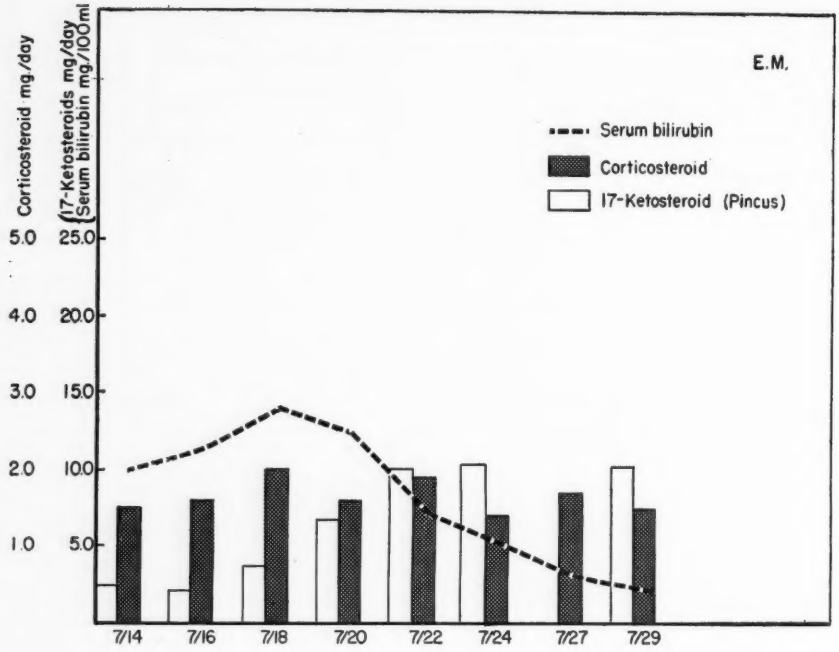

Fig. 3.

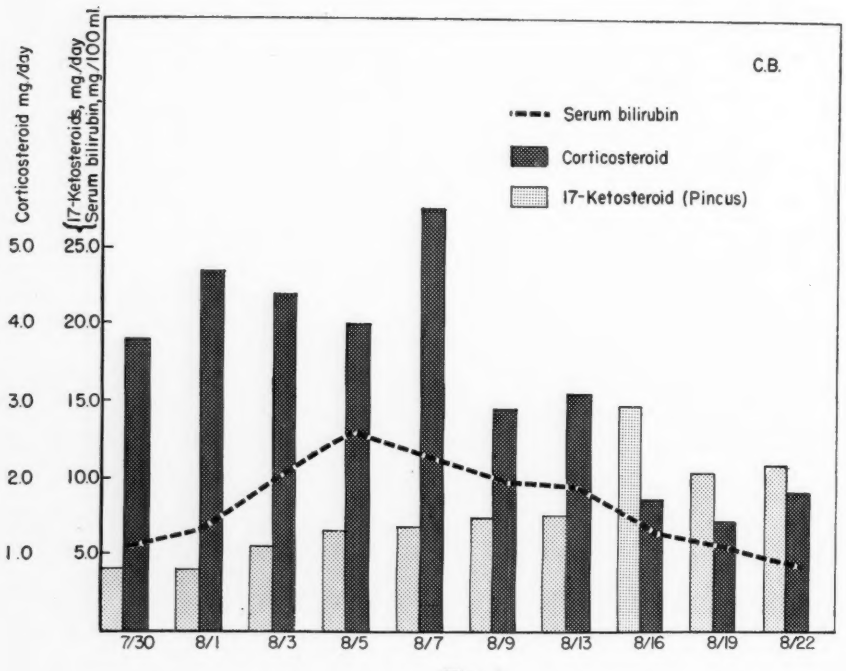

Fig. 4.

Figs. 3, 4, 5, and 6.—Pattern of serial 17-ketosteroid and corticosteroid excretion in representative cases of acute viral hepatitis. (The days of the month are represented along the horizontal.)

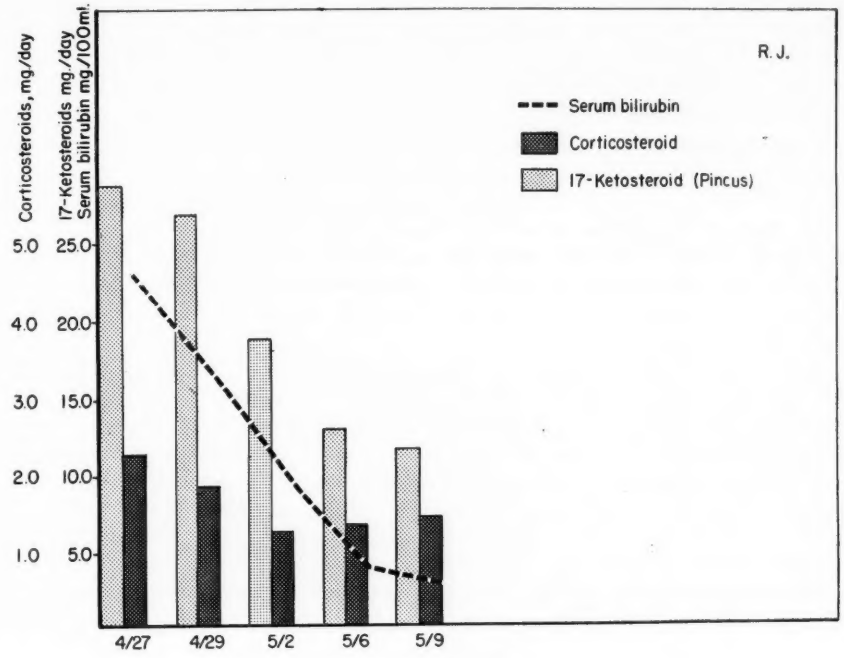

Fig. 5.

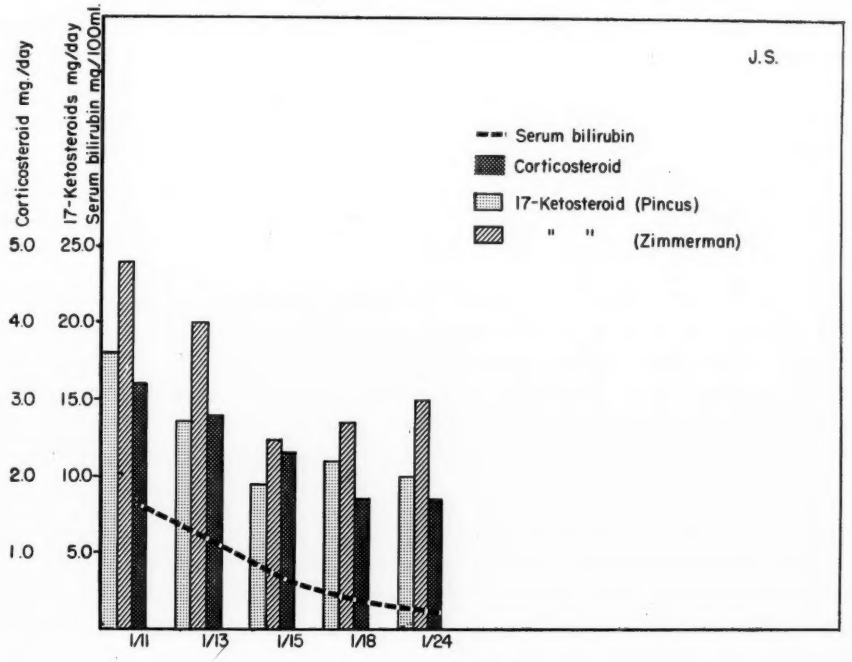

Fig. 6.

Figs. 5 and 6. *(For legends, see opposite page.)*

Twenty-four-hour urines were collected, without preservation, on a metabolic ward and refrigerated. All urines were processed six to twenty-four hours after completion of the collection, treated with zinc acetate,[22] and the filtrate from the zinc acetate precipitation was refrigerated until the assay was begun. The zinc acetate filtrate can be readily extracted with chloroform without the formation of emulsions and preserves the urinary steroids at 4° C. for many months.[23] In 16 cases of acute hepatitis treated with ACTH given by daily intravenous infusions over an eight-hour period, and 22 cases not treated with ACTH, the 17-ketosteroids and the corticosteroids were determined every second or third day throughout the active stage of the disease or until a constant level had been reached. During the early stages of the disease these determinations were made daily in most of the cases. A similar procedure was followed in the patients with chronic hepatitis treated with ACTH. In the patients with cirrhosis, obstructive jaundice, and in the normal persons, two or more determinations were carried out.

The 17-ketosteroids were determined by a modification of the method of Holtorff and Koch,[24] utilizing the *m*-dinitrobenzene (Zimmerman) reaction, and the method of Pincus,[25] utilizing antimony trichloride. With the former, normal adult values approximate 0.1 mg. per pound of body weight. With the Pincus procedure the values range 10 to 20 per cent lower. The formaldehydogenic corticosteroids were determined by a modification[23] of the procedure of Corcoran and Page,[26] using a factor of 11.5 as suggested by Mason[27] to convert formaldehyde to corticosteroid. Normal values (0.7 to 2.5 mg. per day) with this modification more nearly approximate the normals reported by Bassil and Hain.[28] The serum bilirubin method was that of the Ducci and Watson[29] modification of the method of Malloy and Evelyn.[30]

RESULTS

Fig. 1 shows the 17-ketosteroid levels in normal persons, in patients with chronic hepatitis, portal cirrhosis, obstructive jaundice, and early acute viral hepatitis. In the latter condition, these represent the value that deviated most from the patients' own convalescent normal. In many, the 17-ketosteroid levels were lowered just prior to maximal bilirubinemia. In a number of the cases shown, the Zimmerman and the Pincus methods were done on the same specimens. In all but two the Pincus method resulted in slightly lower values.

Fig. 2 shows the results of the urinary formaldehydogenic corticosteroids in normals, in patients in the early active phase of acute hepatitis, chronic hepatitis, portal cirrhosis, and obstructive jaundice.

Figs. 3 and 4 demonstrate the more frequently observed serial urinary 17-ketosteroid pattern as seen in two cases of acute hepatitis. This pattern was observed in 14 of 22 patients not treated with ACTH. Figs. 5 and 6 show the more unusual course. This pattern was observed in only 4 of the 22 patients. In four patients there were no significant abnormalities noted in 17-ketosteroid excretion (in these the maximum serum bilirubin was greater than 10 mg. per 100 ml.).

In only five cases of acute hepatitis were there abnormal elevations of corticosteroids. These reverted to normal with recovery (viz., Figs. 4 and 6). Several of the patients with severe cirrhosis of the liver excreted increased amounts of corticosteroids, and when clinical improvement took place the corticosteroids returned toward normal. Two patients with cirrhosis of the liver developing hepatic coma showed a progressive increase in the corticosteroid excretion.

Five of 16 patients with acute hepatitis given intravenous ACTH showed the general type of urinary 17-ketosteroid and corticosteroid response given by the patient represented in Fig. 7. Seven of the 16 patients gave a type of response represented by the patient shown in Fig. 8, and Fig. 9 is an example of the pattern given by 4 patients. In addition, Fig. 9 represents the type of response obtained in all but one of 8 cases of chronic hepatitis similarly treated, and also is the type of response normally obtained.[31]

Table I lists the urinary steroid data on the 16 patients with acute hepatitis and the 8 patients with chronic hepatitis, before and after infusions of ACTH.

TABLE I. STEROID EXCRETION VALUES IN PATIENTS WITH HEPATITIS GIVEN DAILY INFUSIONS OF 10 TO 20 MG. ACTH

CASE NO.	17-KETOSTEROIDS MG./DAY		CORTICOSTEROIDS MG./DAY	
	BEFORE ACTH*	AFTER ACTH†	BEFORE ACTH*	AFTER ACTH†
Acute Hepatitis				
Group I				
No. 1 (Fig. 8)	–	6.0	–	12.0
No. 2	1.8	4.0	2.0	15.8
No. 3	3.0	5.3	2.5	13.0
No. 4	3.5	6.5	2.5	17.0
No. 5	2.5	4.6	0.8	9.6
No. 6	7.0	7.8	1.5	12.0
No. 7	4.1	7.0	0.6	14.0
Mean	3.7	6.9	1.7	13.1
Group II				
No. 1 (Fig. 7)	17.0	22.5	2.0	9.2
No. 2	14.0	14.7	1.5	10.6
No. 3	10.0	12.8	1.8	13.5
No. 4	11.5	14.0	2.2	16.5
No. 5	15.5	19.7	2.0	12.0
Mean	13.6	16.7	1.9	12.4
Group III				
No. 1 (Fig. 9)	7.0	30.0	1.5	24.0
No. 2	10.0	30.0	0.5	12.3
No. 3	8.8	21.6	0.8	13.5
No. 4	7.3	24.8	1.9	9.8
Mean	8.3	26.6	1.2	14.9
Chronic Hepatitis				
No. 1	10.3	30.0	2.2	12.0
No. 2	19.5	23.4	2.8	20.5
No. 3	17.0	36.0	0.7	9.0
No. 4	12.0	28.0	1.5	17.0
No. 5	8.5	24.0	1.5	14.0
No. 6	11.5	32.0	2.0	11.9
No. 7	11.5	26.7	2.2	11.5
No. 8	15.0	30.2	1.5	12.5
Mean	13.2	28.8	1.8	13.6

*Average of steroid excretion values prior to ACTH infusion.

†Maximum steroid excretion value recorded on fourth or fifth (highest value) day following start of ACTH infusions.

DISCUSSION

Most investigators have reported a very low urinary 17-ketosteroid excretion in acute viral hepatitis,[1, 2, 8, 9, 12] toxic hepatitis,[9] portal cirrhosis,[1, 3, 5, 7-15] biliary cirrhosis,[4, 11] and obstructive jaundice.[1, 8, 9] In general, the results of the present

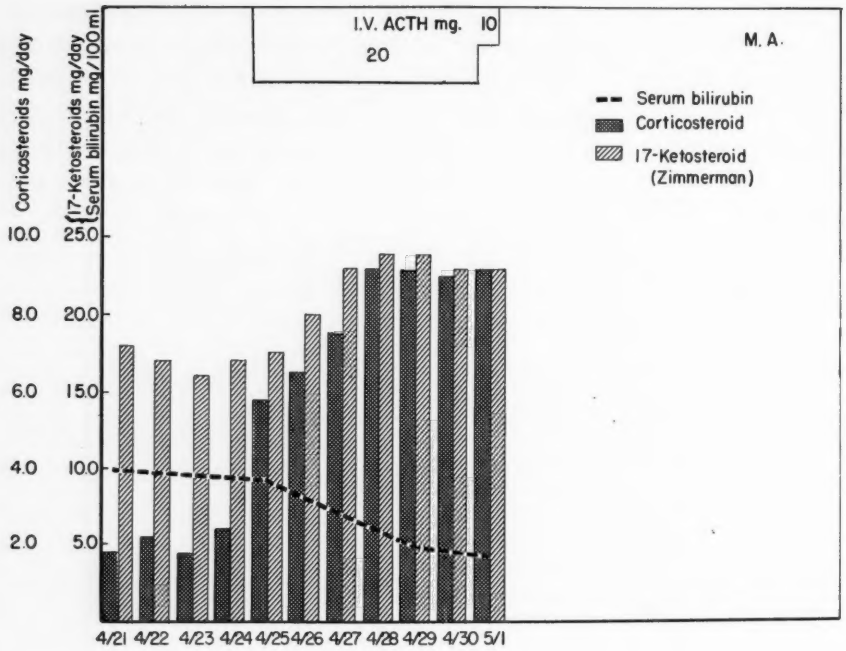

Fig. 7.

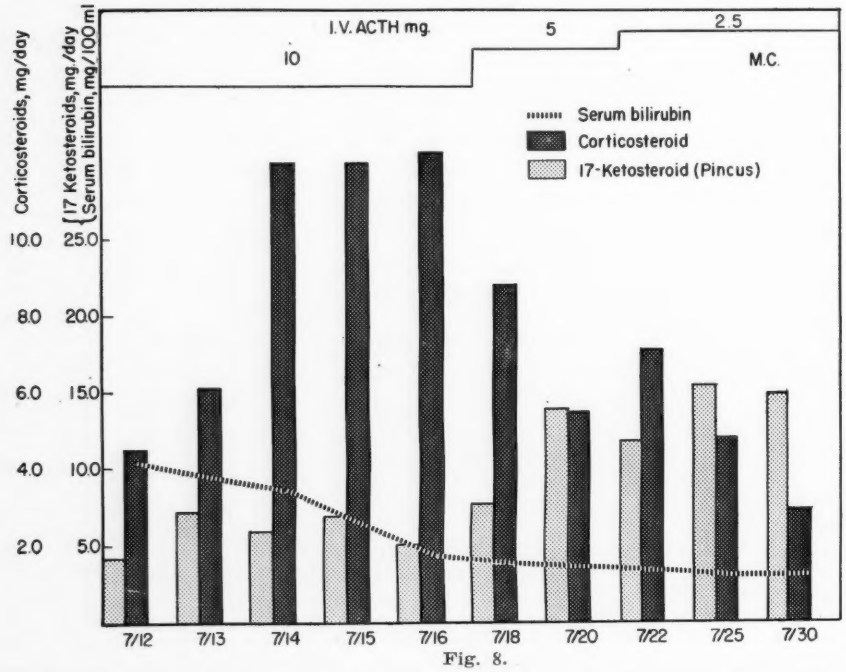

Fig. 8.

Figs. 7, 8, and 9.—Pattern of serial 17-ketosteroid and corticosteroid excretion in representative cases of acute viral hepatitis treated with ACTH. (The days of the month are represented along the horizontal.)

studies confirm these findings. However, with serial studies of 17-ketosteroid excretion in a large number of patients with acute hepatitis it was noted that some patients with severe hepatic disease showed normal, and some increased, urinary excretion of 17-ketosteroids. In the latter group, these high levels returned to normal with recovery.

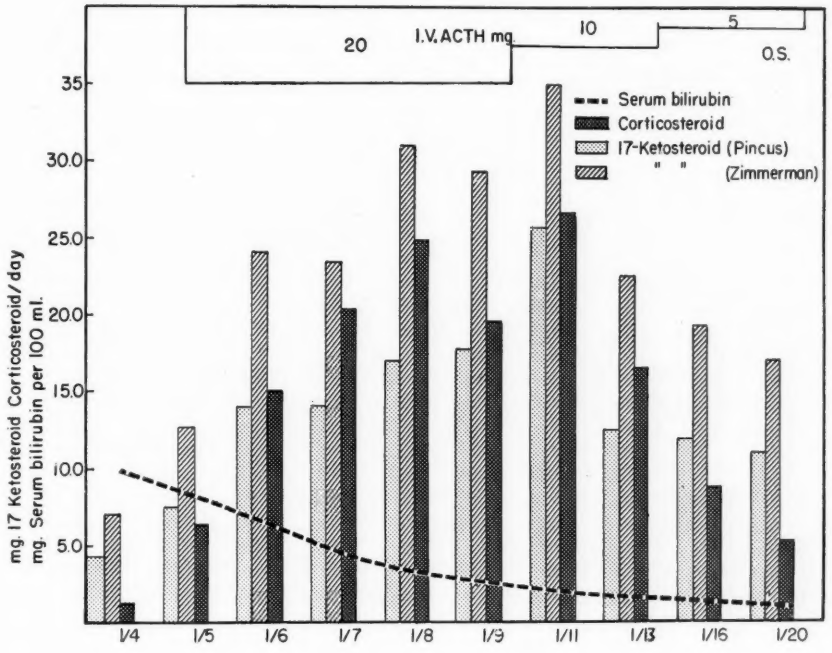

Fig. 9.—*(For legend see opposite page.)*

In a few cases of early obstructive jaundice (carcinoma of the common bile duct and common bile duct stone), the values for the urinary 17-ketosteroids were found in the low range usually seen in hepatitis and cirrhosis. This is in accord with the observations made by other investigators.[1, 8, 9] This finding makes it difficult to explain the lowered 17-ketosteroid values as due entirely to impaired liver cell function. (Two cases of moderately severe hemolytic icterus with normal liver function tests showed normal 17-ketosteroid and corticosteroid values.)

The corticosteroid values in patients with acute hepatitis in nearly all cases fell within the normal range. In five cases, however, they were slightly elevated and returned to normal with recovery. In the six cases of obstructive jaundice studied, no abnormalities of corticosteroid excretion were noted. The finding of an increased corticosteroid excretion in cirrhosis of the liver confirms the results of other observers.[8, 10, 11, 14] These high values have been noted to return to normal with improvement of the disease and to become more elevated in two patients that developed coma and died.

In both acute hepatitis and cirrhosis there did not seem to be any consistent relationship between the levels of 17-ketosteroid and the corticosteroids.

It has been shown that there are at least three different urinary 17-ketosteroid response curves following ACTH infusions in acute viral hepatitis. In one group (Fig. 9) the response was the same as observed in the "controls" (patients with chronic hepatitis, and normals[31])—increased excretion of 17-ketosteroids and corticosteroids following daily ACTH stimulation. In a second group (Fig. 7) the urinary 17-ketosteroids were not increased or were very much less than observed in the "controls," and remained at a fairly constant level during and following ACTH stimulation. In the third group (Fig. 8) the rise in 17-ketosteroids following ACTH was only slight initially, but with clinical improvement and decreased ACTH dosage there was a rise to normal levels. In all three groups the corticosteroid excretion appeared to be within the range seen in normals[31] and mild chronic hepatitis cases given intravenous ACTH, and thus there was no suggestions of a decreased adrenal responsiveness. Bongiovanni and Eisenmenger[11] have reported an apparently normal adrenal response to ACTH in patients with cirrhosis.

Some of the mechanisms that have been suggested to explain the low level or urinary 17-ketosteroids frequently seen in acute hepatitis and other forms of liver and biliary tract disease are: (a) decreased production of pituitary adrenocorticotrophin, (b) decreased pituitary gonadotropic hormone production, (c) decreased conversion of testosterone or other testicular androgen to 17-ketosteroids, (d) depressed adrenal cortical function, and (e) diminished conversion of corticosteroids to 17-ketosteroids.

The presence of a normal or increased excretion of urinary corticosteroids is evidence against the concept of a decreased production of endogenous ACTH.

Some workers have reported a decrease in excretion of pituitary gonadotropic hormone in liver disease.[5, 7, 14] Others, however, have not found this to be the case.[12] The depression of the 17-ketosteroid excretion seems too great to be explained on the basis of only a decreased production of a testicular androgen alone, since most of the 17-ketosteroids arise as a result of adrenal activity. Some investigators have reported a decreased conversion of exogenous testosterone to 17-ketosteroids in liver disease.[3, 18] Others[6, 11, 13] have disputed this finding. Despite a supposed decrease in the conversion of endogenous testosterone to 17-ketosteroids, the marked depression of the urinary 17-ketosteroids seen in some cases of liver disease could not be explained on this basis.

The presence of a normal or increased corticosteroid excretion would be inconsistent with the concept of hypoadrenalism. The normal corticosteroid response to ACTH is further evidence against such a concept.

The administration of 17-hydroxylated corticosteroids or 10 mg. or more of ACTH intravenously over an eight-hour period to normal persons causes a marked rise in 17-ketosteroids.[31] (The 17-ketosteroids reach a plateau after three to five days, whereas the corticosteroids reach a plateau after two to four days.) Butt and associates[32] have shown that when intramuscular cortisone acetate was given to two patients with parenchymal liver disease, there was no increase in the urinary 17-ketosteroids. Conn[33] has reported that a much smaller

increase in urinary 17-ketosteroids results when 11-desoxy-17-hydroxycorticosterone (Compound S) is given orally to patients with cirrhosis than in normal persons. The results of the present study (Figs. 7 and 8) are in line with these observations (minimal increase in urinary 17-ketosteroids during active stage of acute hepatitis in face of normal levels of corticosteroids in 12 of 16 patients given intravenous ACTH); however, we have produced the high level of corticosteroids endogenously, through ACTH stimulation. These studies lend credence to the concept of a decreased conversion of corticosteroids to 17-ketosteroids by the liver in acute hepatocellular liver disease (acute viral hepatitis). The low levels of 17-ketosteroids seen in obstructive jaundice as well as in parenchymal liver disease are not, however, readily explained by a diminished conversion of corticosteroids to 17-ketosteroids by the liver.

Bongiovanni and Eisenmenger[11] have suggested that the low levels of urinary 17-ketosteroids may be related to the low urine volumes found in liver disease. In this study no such relationship was noted.

It is entirely possible that the few instances where high levels of urinary corticosteroids were found do not actually represent increased excretion of corticosteroids but rather increase in the unconjugated forms of corticosteroids. This is possibly due to failure of conjugation by the liver since the method for the formaldehydogenic corticosteroids used in this study does not measure total corticosteroid excretion.

SUMMARY

Patients with acute infectious hepatitis, portal cirrhosis, and obstructive jaundice have been found in most instances to have a low 17-ketosteroid excretion. The majority of the patients with early acute hepatitis studied with serial twenty-four-hour urine collections were found to have low 17-ketosteroids initially, followed by return to normal during recovery. A small number, however, did not demonstrate this deviation and maintained normal 17-ketosteroid excretion, while a few showed a definite increased excretion during the early phase of the disease. A good correlation was noted when the 17-ketosteroids were determined either by the Pincus method or by the Zimmerman procedure, though the latter gave slightly higher values.

Patients with moderately severe and severe cirrhosis showed increased corticosteroid (formaldehydogenic) excretion. Only five acute hepatitis cases showed abnormally elevated corticosteroids when studied serially. These returned to the normal with recovery.

Three different patterns of urinary 17-ketosteroids, associated with a normal corticosteroid excretion, have been observed following ACTH stimulation in patients with acute viral hepatitis: (1) normal response (increased excretion), (2) no increase or only minimal increased excretion in active and convalescent phases of disease, and (3) continued low level of 17-ketosteroids during active phase of disease, followed by a delayed rise to normal levels with recovery. It is suggested that the most plausible explanation for this minimal 17-ketosteroid response following ACTH stimulation is a diminished conversion of corticosteroids to 17-ketosteroids by the liver in acute hepatitis.

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AN EVALUATION OF THE EFFECTS OF CORTISONE ON THE SUBCUTANEOUS NODULES OF PATIENTS WITH RHEUMATOID ARTHRITIS

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THE subcutaneous nodule, when it is present, embodies the most distinctive histologic picture of any of the lesions in patients with rheumatoid arthritis.[12] The approximate incidence of this lesion has been reported as 10 per cent.[1-3] Several investigators have observed a decrease in the size of these nodules during cortisone and ACTH administration.[13, 15, 16, 17] However, histologic examination before, during, and after the administration of these hormones disclosed no change in the structure of these nodules.[4-8] Definite changes in the histologic structure of the nodules during and after cortisone administration were reported by others.[9, 10, 11, 14, 15, 17]

This study was undertaken to gain further information concerning the possible influence of cortisone upon established nodules in patients with rheumatoid arthritis.

METHODS

Selection of Patients.—Eight patients with rheumatoid arthritis who had bilaterally symmetrical subcutaneous nodules of similar dimensions were selected. All patients had nodules either in the olecranon or forearm areas, or in both sites. Three of these patients also had multiple nodules on the hands, knees, and Achilles tendon areas.

There were five female and three male patients. One man and one woman were Negro; the remainder were white. Their ages ranged from 41 to 61 years, and all had chronic, active rheumatoid arthritis (Table I).

Procedure of Nodule Measurement.—In six patients, the nodules were measured and one was removed as the control specimen (Fig. 1). In two patients, the initial (control) nodule was removed in another hospital from which the slides were made available to us for study. A plaster of Paris mold of the opposite symmetrical nodule was then constructed prior to the administration of cortisone (Fig. 2). Upon completion of the steroid administration, a second mold of the same nodule was constructed (Fig. 3), and this nodule was then excised. The plaster of Paris molds of the nodules were then measured volumetrically. The length, width, and height of the nodule imprint were also recorded. The periphery of the imprint of each nodule was delineated on the mold with a soft lead pencil and the entire surface was impressed in modeling clay. When the mold was removed, the pencil markings were imbedded in the clay. This negative impression was filled to the pencil line with water from a calibrated syringe. The quantity of fluid necessary to fill the depression represented the volume of the nodule. This casting procedure was completed in five of the six patients in the first series studied; in the remaining patient, linear measurements of the nodule were recorded.

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Dosage and Duration of Cortisone Administration.—Cortisone, 100 mg., was administered intramuscularly once daily, six days a week. The hormone was given to one patient for eight days, to two for twenty days, to one for twenty-two days, to one for twenty-six days, and one patient had two separate courses. For this patient, the first course was of twenty days' duration, when one nodule was excised; the second course was of sixty days' duration, when another nodule was excised. Two patients received the drug for twelve and thirteen months, respectively. (These two patients were those in whom the control nodules were removed elsewhere prior to the administration of cortisone.)

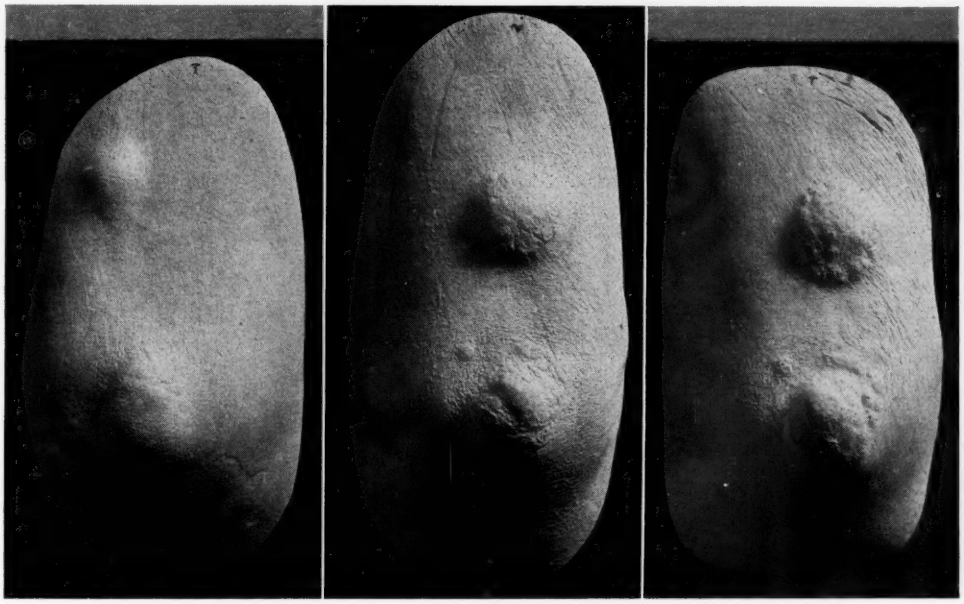


Fig. 1. Fig. 2. Fig. 3.

Fig. 1.—Plaster of Paris mold of subcutaneous nodule excised for control purposes prior to cortisone administration (Case 5).

Fig. 2.—Plaster of Paris mold of subcutaneous nodule before cortisone administration (compare with Fig. 3). (Case 5.)

Fig. 3.—Plaster of Paris mold of subcutaneous nodule (see Fig. 2) after twenty-two days of cortisone administration. (Case 5.)

RESULTS

Duration of Arthritis in Relation to the Appearance of the Subcutaneous Nodules.—In the patients studied, rheumatoid arthritis had existed for from two to twenty-nine years prior to the onset of the subcutaneous nodules. In six patients the nodules had been present for from six months to two years, and in two patients for five and sixteen years, respectively (Table I). The appearance of the nodules bore no apparent relationship to the stage and activity of the disease. All the patients were ambulatory.

Effect of Cortisone on the Consistency and Size of the Subcutaneous Nodules.—There was detectable softening of all the nodules following the use of cortisone. In four patients to whom cortisone was given for short periods of time, this change was noted within fourteen days after its initiation, and in the remaining two patients softening was observed after

TABLE I. CLINICAL DATA ON PATIENTS STUDIED

CASE	AGE	SEX	RACE	DURATION OF ARTHRITIS (YR.)	CLASSIFICATION OF ARTHRITIS*	DURATION OF NODES (YR.)
1	42	F	W	4	Stage 2 Class 2	1
2	44	F	W	9	Stage 2 Class 2	1
3	41	F	N	10	Stage 2 Class 2	½
4	45	F	W	16	Stage 3 Class 2	5
5	52	M	W	5	Stage 3 Class 2	2
6	61	M	N	31	Stage 3 Class 3	2
7	40	M	W	2	Stage 2 Class 3	1
8	45	F	W	16	Stage 3 Class 1	16

*American Rheumatism Association; Steinbrocher's Therapeutic Criteria.

TABLE II. DIMENSIONAL AND VOLUMETRIC MEASUREMENTS OF NODULES BEFORE AND AFTER CORTISONE ADMINISTRATION

CASE	DIMENSIONS BEFORE CORTISONE (MM.)	DIMENSIONS AFTER CORTISONE (MM.)	VOLUME BEFORE CORTISONE (ML.)	VOLUME AFTER CORTISONE (ML.)	PER CENT OF CHANGE IN VOLUME
1	18 × 23 × 7	18 × 22 × 4	1.25	0.60	−56
2	28 × 24 × 9	26 × 23 × 6	1.62	1.35	−17
3	20 × 20 × 10 16 × 20 × 5	20 × 15 × 10 16 × 20 × 3	0.92	0.56	−40
4	15 × 15 × 10 30 × 25 × 15	12 × 12 × 5 25 × 23 × 10			
5	29 × 26 × 11 28 × 26 × 8	29 × 26 × 10 30 × 27 × 8	4.00 3.45	4.25 4.05	+5 +16*
6	24 × 35 × 11	25 × 32 × 8	3.20	2.76	−14

*Not studied histologically.

seven and twenty-one days, respectively. Change in the size and volume of the nodule was concomitant with softening. Due to the irregularity of the nodule surface the plaster of Paris mold provided a more accurate means of ascertaining the change in size of a given nodule during cortisone administration than did direct measurement of the specimen.

It will be noted in Table II that only two nodules (Case 5) of the nine specimens examined failed to show a measurable reduction in size either by direct or volumetric measurement. In this patient, two nodules existed in close proximity, one showing a slight increase in size (15 per cent), and the other, which was not excised for microscopic study, a measurable increase in size (16 per cent).

Of the six nodules measured volumetrically, four showed a reduction of from 14 to 56 per cent. Failure to obtain volumetric measurements on six of the specimens was due in one case (Case 3) to the location of the lesion, a position on the hand which prevented accurate casting. In the other five instances (Cases 4, 7, and 8) the artist who prepared the molds was not available when these patients came under observation.

Comparison of the data recorded in Table II indicates that the chief difference in the dimensions of the nodules following cortisone administration was in the thickness of the lesion. Reduction in this dimension may have been the result of the increased plasticity or softening of the lesion. This change also may have accounted for the shallower depressions in the mold and thus may be the principal cause of the observed reduction in volume of the mold imprints.

Fig. 4.

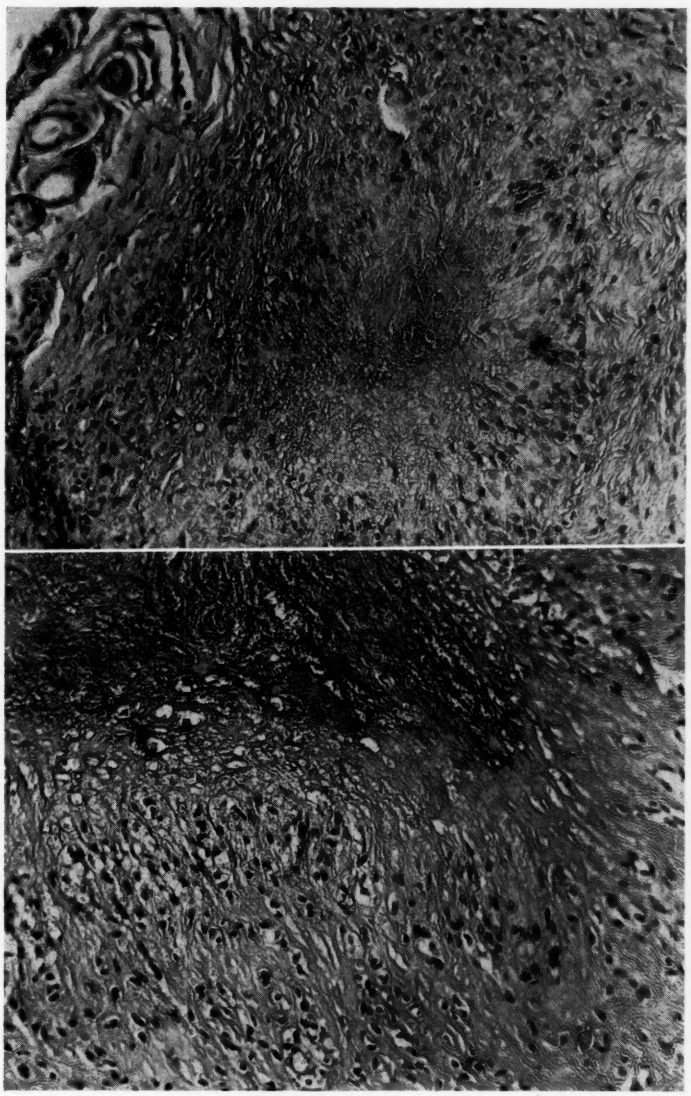


Fig. 5.

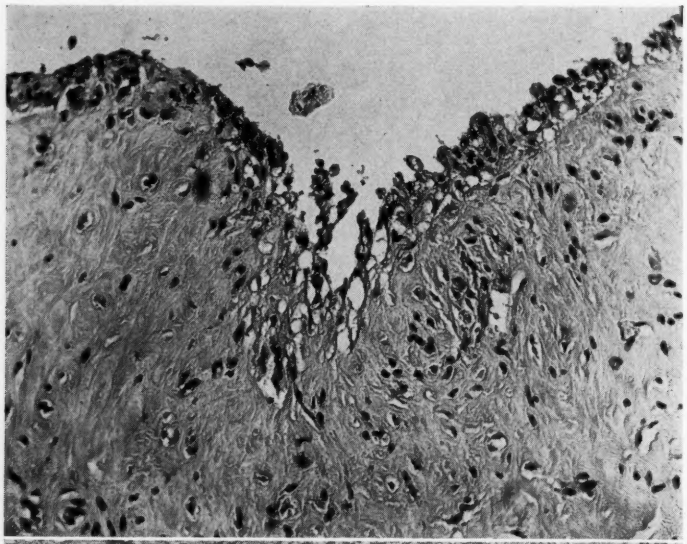
Fig. 4.—Control nodule showing moderate necrosis and palisading of marginal cells. (Case 1, left, Table III.) (×230; reduced ⅓.)

Fig. 5.—Nodule removed after administration of cortisone for eight days. Necrosis and palisading are greater than that shown in Fig. 4. (Case 1, right, Table III.) (×230; reduced ⅓.)

Preparation of Specimens for Pathologic Study.—Histologic studies were made on 20 specimens representing eight pairs of nodules from six patients in the first group observed, one additional nodule from Case 2 after a second and longer course of cortisone administration, and three nodules from two patients who received cortisone for twelve and thirteen months, respectively.

The nodules were excised under local (Novocain) anesthesia, with care to minimize trauma to the specimen. Techniques were employed which permitted the use of the

Fig. 6.



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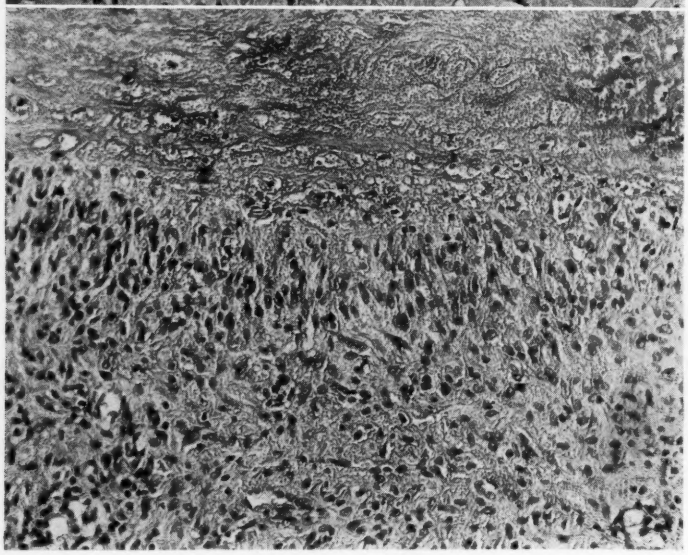

Fig. 6.—The margin of a cystic space is illustrated in this inactive appearing nodule removed before cortisone. (Case 3, left, Table III.) (×230; reduced ⅓.)

Fig. 7.—Marked necrosis and palisading of cells are illustrated in this lesion twenty days after cortisone was administered. (Case 3, right, Table III.) (×230; reduced ⅓.)

following staining methods: hematoxylin and eosin, eosin-methylene blue, Mallory's connective tissue stain, phosphotungstic acid-hematoxylin, toluidine blue and periodic-acid Schiff reaction.

The stained sections were first examined without reference to patient identification or to whether or not cortisone had been administered. On a basis of arbitrarily selected criteria, an attempt was made to grade on a scale of 0 to 4-plus the outstanding histologic qualities, including fibrous encapsulation, cystic degeneration, inflammation, palisading of cells, and necrosis in each specimen. The recorded data were then charted for comparison of the nodule which was removed from a given patient before cortisone was administered with the paired nodule excised after cortisone had been injected.

Fig. 8.

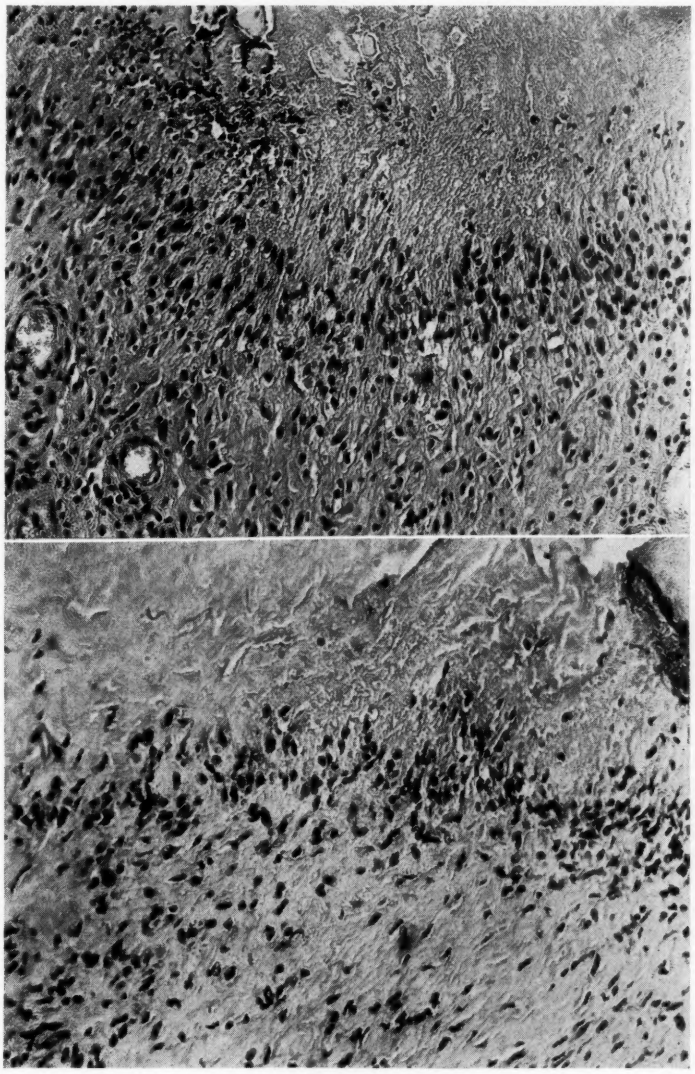

Fig. 9.

Figs. 8 and 9.—These photomicrographs illustrate only minor microscopic differences between the control and the postcortisone nodules removed from the right (Fig. 8), and left (Fig. 9), elbows of Case 4. (×230; reduced ⅓.)

TABLE III. HISTOLOGIC CHANGES OBSERVED IN NODULES REMOVED BEFORE AND AFTER ADMINISTRATION OF CORTISONE

CASE	1		2			3		3		4		4		5		6		7	8	
Location of nodule	L	R	L	R	L	L	R	L	R	R	L	R	L	R	L	R	L	R	L	R
Number days on cortisone	0	8	0	20	60	0	20	0	20	0	20	0	20	0	22	0	26	365	0	395
Encapsulation	++	++	+++	++	++++	++++	+++	+++	++	++	++	++	++	++++	++	++++	+++	+	++	+
Inflammation	+	+	++	++	+	++	++	++	++	++++	+++	+++	+++	++	++	+	++	+++	++	++
Cystic degeneration	0	0	0	0	0	+++	+++	+++	+	0	0	0	+	++++	+	++++	+++	0	±	0
Necrosis	+++	++++	+++	+++	+	+++	++++	++	+++	++++	++++	++++	++++	+	++++	++++	++++	++++	+++	++++
Palisading	+	++	+++	+++	+	++	++++	++	+++	++++	++++	++++	++++	+++	++	++	+++	++++	+++	+++
Over-all activity	++	++++	+++	++++	+	+++	++++	++	+++	++++	++++	++++	++++	+	+++	+++	+++	++++	+++	+++

Histologic Findings in Nodules Removed Before and After Cortisone Administration.—Pathologic examination of the 20 specimens demonstrated that all possessed the typical microscopic features of the subcutaneous nodule of rheumatoid arthritis (Figs. 4-9).[12] Although there was considerable variation in microscopic composition between individual specimens from the same and different patients, there was reasonable similarity in the over-all structure of the nodules removed after cortisone was started and the paired nodule removed subsequently (Table III).

In Case 1, in the nodule which was removed eight days after the beginning of cortisone, there was greater necrosis and palisading and greater over-all activity than was observed in the nodule removed before cortisone was administered. The nodule removed twenty days after cortisone administration in Case 2 showed somewhat greater over-all activity and lessened encapsulation than did the nodule removed before cortisone was given.

Two sets of nodules were compared in Case 3. In the first pair, there was lessened encapsulation along with greater degrees of necrosis, palisading, and over-all activity in the nodule removed after twenty days of cortisone injection. In the second pair of lesions from this patient a similar relationship was seen to exist.

In Case 4, two sets of nodules were compared. In both pairs the findings were essentially alike in the lesions removed before cortisone was given and in the paired nodules excised after twenty days of cortisone injection.

In Case 5, encapsulation and palisading were less prominent, but necrosis and over-all activity were much greater in the postcortisone nodule than in the initial lesion.

In the nodule removed twenty-six days after cortisone was started (Case 6), there was lessened encapsulation and greater inflammation and palisading than was noted in the control nodule removed before drug administration. In over-all activities, however, the two lesions appeared similar.

Thus it will be noted that in five instances the nodule which was removed after cortisone was administered appeared more active than did the paired nodule which was removed before cortisone was given. In the three remaining pairs of specimens (Cases 4 and 6), there were no observable differences.

In three patients nodules were excised after longer periods of cortisone administration. These periods were two, twelve, and thirteen months in duration. In Case 2, the nodule removed twenty days after cortisone was started appeared somewhat more active than did the nodule removed before the drug was given. However, the second nodule, excised two months after the initiation of a second course of cortisone, showed pronounced fibrosis and very little histologic evidence of activity. The patient designated Case 7 received cortisone and ACTH for one year prior to the excision of a typical and active appearing nodule. Although the lesion was not observed at the

beginning of the patient's treatment, its presence was recognized during the final six months of hormone administration. In Case 8, the nodules removed before, and thirteen months after, administration of cortisone appeared to be identical on histologic examination.

SUMMARY

In this study we have made clinical and histologic observations on rheumatoid nodules in eight patients to whom cortisone was administered for periods ranging from eight days to thirteen months. It is concluded that cortisone administration may induce palpable softening of the subcutaneous nodule, but that within the time periods of this study, the hormone fails to induce any consistent modifications in the principal histologic patterns of the lesions.

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IMPORTANCE OF THE LIVER IN TRANSFORMATIONS OF ADMINISTERED ADRENOSTEROIDAL COMPOUNDS

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IN THE course of a series of studies on the metabolic effects of various adrenal steroids in human subjects, it became apparent that the level of urinary excretion of 17-ketosteroids depended in part upon the route of administration of each of the steroids. In a preliminary communication[1] it was suggested (a) that the liver plays a major role in the transformation of the steroids to 17-ketosteroids, (b) that rate of hepatic conversion of administered steroids to 17-ketosteroids is related to the hemic concentration of steroid presented to the liver, and (c) that the damaged liver is incapable of performing this function normally. The purpose of the present report is to record the data from which these conclusions have been derived.

METHODS

Daily renal excretions of 17-ketosteroids[2, 3] and of formaldehydogenic steroids[4] by normal subjects were measured during oral as well as during intramuscular administration of each of five steroidal compounds. The substances used were compound F and compound F acetate (hydrocortisone and its acetate), compound E acetate (cortisone acetate), compound B (corticosterone), and compound S acetate (11-dosoxy-17-hydroxycorticosterone acetate). Comparable excretory studies were performed during oral administration of compound S.

Six students, five males (R. S., G. A., L. W., G. B., and L. B.) and one female (J. B.), served as subjects for the studies in normal individuals. For comparative purposes a given compound was administered both orally and intramuscularly to the same subject, except in the case of cortisone acetate where the comparison was made between two of the males. In each experiment the amount of steroid administered was either 200 mg. or 400 mg. per day. When administered intramuscularly, compounds B, E acetate, and S acetate were given twice daily, and compounds F and F acetate four times daily.† During oral administration of the steroids the total daily amount was divided into three or four doses.

Early in this study it was found that orally administered compound S, despite its lack of metabolic activities, evoked a great increase in urinary 17-ketosteroids.[5] It was decided, therefore, to employ compound S or its acetate for study of the phenomenon in patients with hepatic disease.

DESCRIPTION OF PATIENTS

Five patients (E. J., G. B., N. S., I. R., and E. S.) with moderate to severe impairment of liver function were studied. E. J. and G. B. were cases of Laennec's cirrhosis.

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†We are indebted to the Upjohn Company, Kalamazoo, Michigan, for compounds B, F, S, and S acetate, and to Merck & Company, Rahway, New Jersey, for the compound F acetate used in these studies.

N. S. was chosen for this study because in addition to severe liver disease he had coexisting panhypopituitarism with practically no endogenous production of steroid hormones. In 1951 he had undergone adenohypophysectomy because of the presence of a large chromophobic adenoma. His liver disease had begun with acute hepatitis in 1933, since which time episodes of painless jaundice had appeared frequently. The last recrudescence began in February, 1952, three weeks before the present studies were begun.

I. R. was a patient with chronic hepatitis. Acute hepatitis began in September, 1951. Great clinical improvement occurred in November, 1951, followed in January, 1952, by a severe recrudescence. The renal excretory studies to be reported were obtained in March, 1952, at which time very mild improvement had occurred, and again in September, 1952, when the patient was very much better. Liver function studies done both times are shown in Table I.

TABLE I. LIVER FUNCTION STUDIES ON PATIENT I. R.

	MARCH, 1952	SEPTEMBER, 1952
Total serum bilirubin	13.7 mg. %	1.5 mg. %
Free (1 minute)	5.2 mg. %	0.1 mg. %
Free (direct 30 minutes)	9.5 mg. %	0.5 mg. %
Combined (indirect)	4.2 mg. %	1.0 mg. %
Gamma globulin	20.1 units	9.3 units
Thymol turbidity	6.8 units	2.6 units
Cephalin-cholesterol flocculation 24 hr.	+ + +	+ +
Cephalin-cholesterol flocculation 48 hr.	+ + + +	+ + +
Total serum proteins	6.1 %	7.8 %
Albumin	2.9 %	4.7 %
Globulin	3.2 %	3.1 %
A/G	0.9	1.5
Prothrombin concentration	49 %	100 %
Bromsulphalein retention (45 min.)	—	14.3 %
Total cholesterol	106 mg. %	136 mg. %
Free	56 mg. %	39 mg. %
Esters	50 mg. %	97 mg. %
Per cent esters	48	71

E. S. was a patient with hemochromatosis and marked hepatomegaly.

A sixth patient, F. H., a well-documented case of Addison's disease, in whom liver function was normal, was studied in the same way.

RESULTS

Figs. 1 and 2 compare the effects of intramuscular versus oral administration of free compound F on urinary excretion of 17-ketosteroids and formaldehydogenic steroids. When 400 mg. per day of compound F were given intramuscularly (Fig. 1) urinary 17-ketosteroids decreased slightly at first and then increased mildly. On the other hand, there occurred a fivefold increase in excretion of formaldehydogenic steroids. In contrast when free compound F was given *orally* in the same quantity (Fig. 2) there occurred a rapid and large increase in the excretion of 17-ketosteroids. Urinary formaldehydogenic steroids also increased greatly, but somewhat less than during intramuscular administration of compound F. The over-all metabolic effects of this compound were similar regardless of the route of administration.[6]

Fig. 3 shows the effects of daily administration of 400 mg. of compound F acetate upon these urinary excretory products. When given intramuscularly

the steroid produced a sharp decrease in 17-ketosteroids and a small increase of formaldehydogenic steroids. During oral administration of the same compound there occurred a great increase in excretion of both 17-ketosteroids and

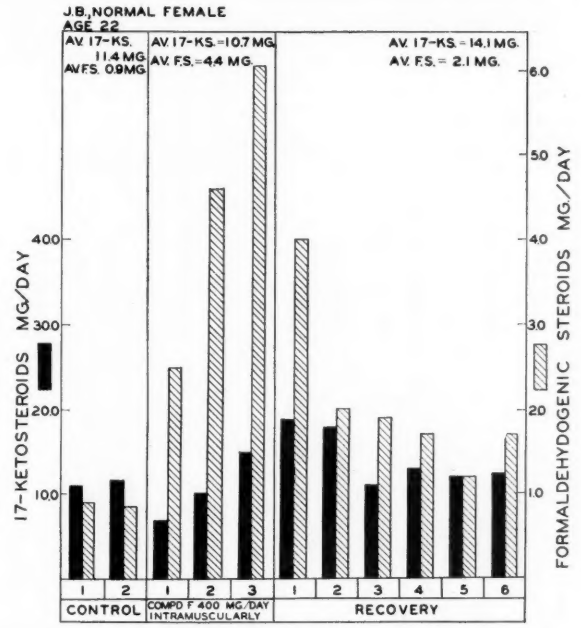

Fig. 1.—Effect of intramuscular free compound F on urinary steroidal excretion.

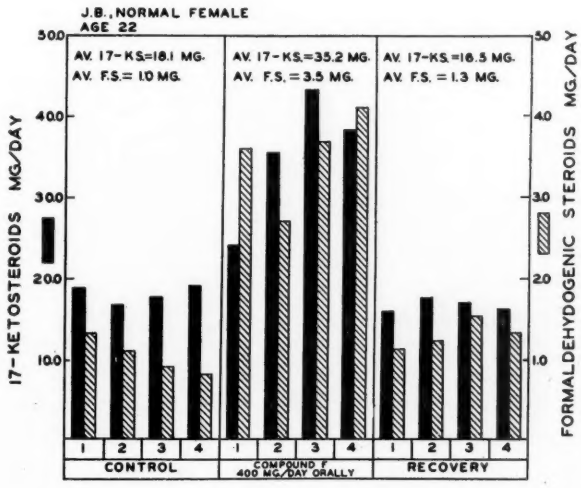

Fig. 2.—Effect on oral free compound F on urinary steroidal excretion.

formaldehydogenic steroids. Oral administration of this compound produced intense metabolic effects, while only minimal metabolic changes occurred upon intramuscular administration.[6]

Table II compares the data obtained during intramuscular and during oral administration of cortisone acetate and of corticosterone. At 200 mg. per day intramuscularly compounds E acetate and B caused small decreases from baseline levels in excretion of 17-ketosteroids. When given orally they produced small increases in urinary 17-ketosteroids. On the other hand, each compound by either route resulted in a small increase of formaldehydogenic steroids.

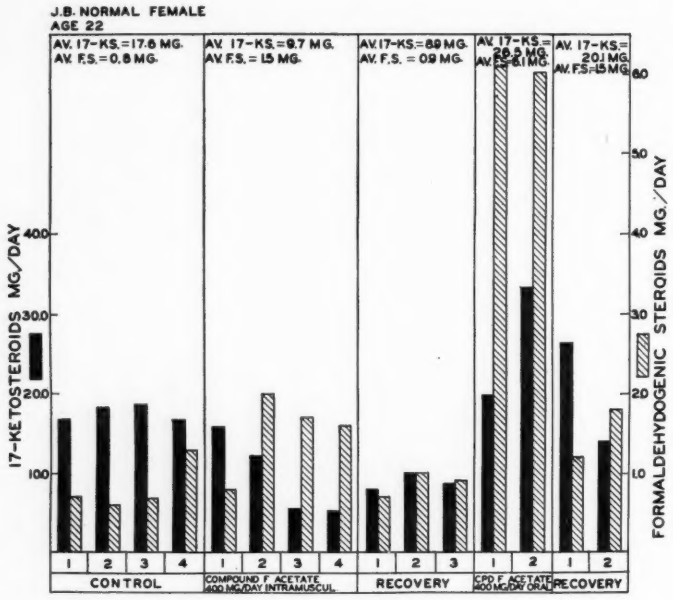

Fig. 3.—Effect of compound F acetate on urinary steroidal excretion.

TABLE II. EFFECT OF CORTISONE ACETATE (COMPOUND E) AND CORTICOSTERONE (COMPOUND B), 200 MG./DAY, ON URINARY STEROIDAL EXCRETION IN NORMAL MALES

SUBJECT	COMPOUND	ROUTE OF ADMINISTR.	17-KETOSTEROIDS MG./DAY			FORMALDEHYDOGENIC STEROIDS MG./DAY		
			CONTROL PERIOD	STEROID ADMINISTR.	RECOVERY PERIOD	CONTROL PERIOD	STEROID ADMINISTR.	RECOVERY PERIOD
R. S.	E	I. M.	16.0(5)	12.7(10)	11.6(16)	1.5(6)	2.1(10)	1.9(16)
G. A.	E	O	15.9(3)	19.4(5)	11.6(3)	1.2(3)	1.7(5)	0.9(1)
G. A.	B	I. M.	15.8(4)	13.9(6)	13.5(2)	1.3(4)	1.9(6)	1.8(2)
G. A.	B	O	13.4(5)	16.5(5)	13.0(4)	1.0(5)	1.5(5)	1.1(4)

Numbers in parentheses denote number of days in each period.

Figs. 4 and 5* show the effects of administration of compound S acetate upon the two classes of excretory steroids. When 200 mg. per day were administered orally to subject R. S. (Fig. 4), there was a great increase in the excretion of 17-ketosteroids which was not seen when the steroid was administered intramuscularly for four days. The increase in formaldehydogenic steroids was minimal by both routes of administration. Fig. 5 indicates that

*Figs. 4, 5, and 6 have been published previously.[5] They are reproduced here with the publisher's permission for purposes of comparison wth the oral and intramuscular administration of other steroids.

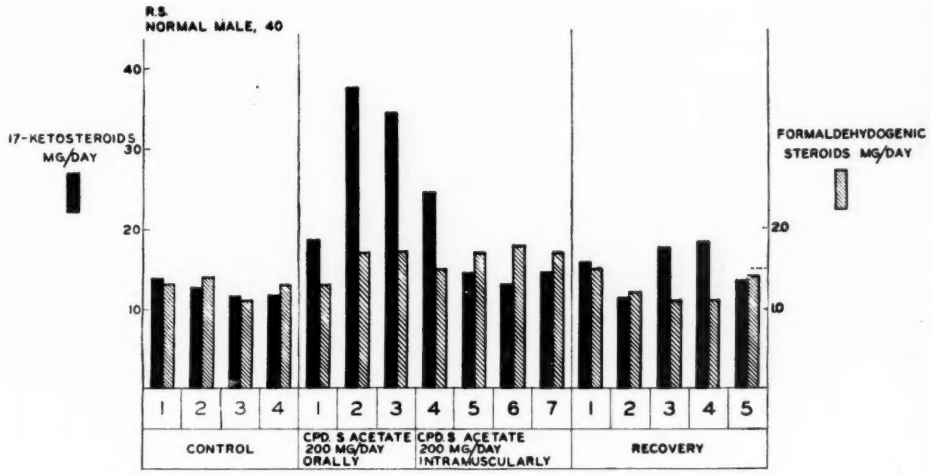

Fig. 4.—Effect of compound S acetate on urinary steroidal excretion.

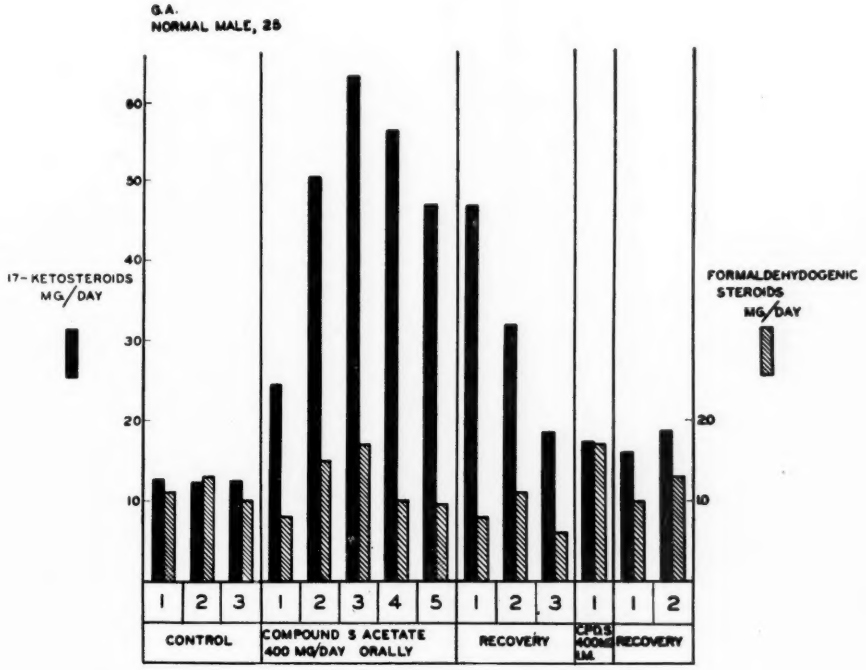

Fig. 5.—Effect of compound S acetate on urinary steroidal excretion.

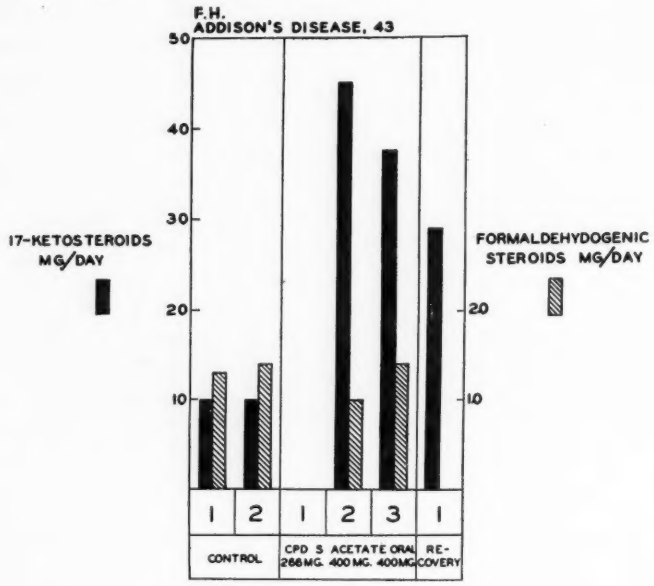

Fig. 6.—Effect of compound S acetate on urinary steroidal excretion.

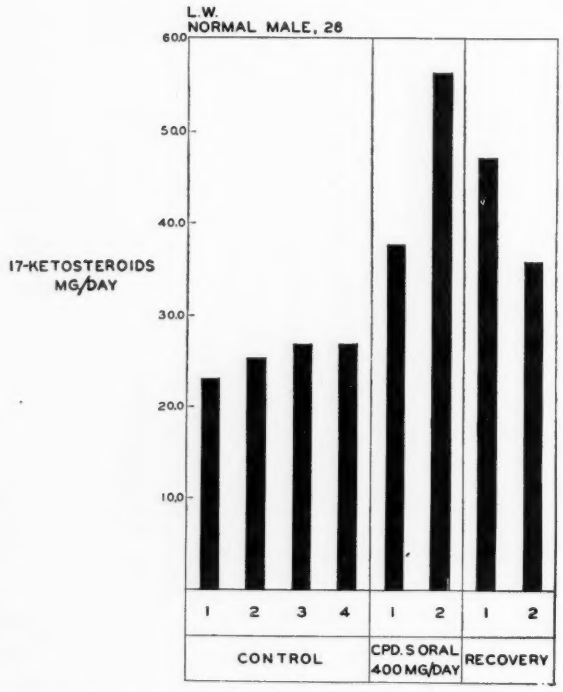

Fig. 7.—Effect of free compound S on renal excretion of 17-ketosteroids.

doubling the oral dose of compound S acetate results in an even greater increase in excretion of 17-ketosteroids (compare with Fig. 4). While 400 mg. of compound S acetate were administered intramuscularly to G. A. for but one day, it produced no increase in the excretion of 17-ketosteroids. The increase in excretion of 17-ketosteroids during oral administration of compound S acetate was demonstrated to be due to an increase in the ketonic fraction.[5] Again, there were no significant changes in excretion of formaldehydogenic steroids during administration of compound S acetate.

Fig. 6 demonstrates that oral administration of compound S acetate to a patient with Addison's disease (F. H.) results in an equally prompt and great rise in excretion of 17-ketosteroids. As in the normal subjects the Addisonian exhibited no significant change in the excretion of formaldehydogenic steroids.

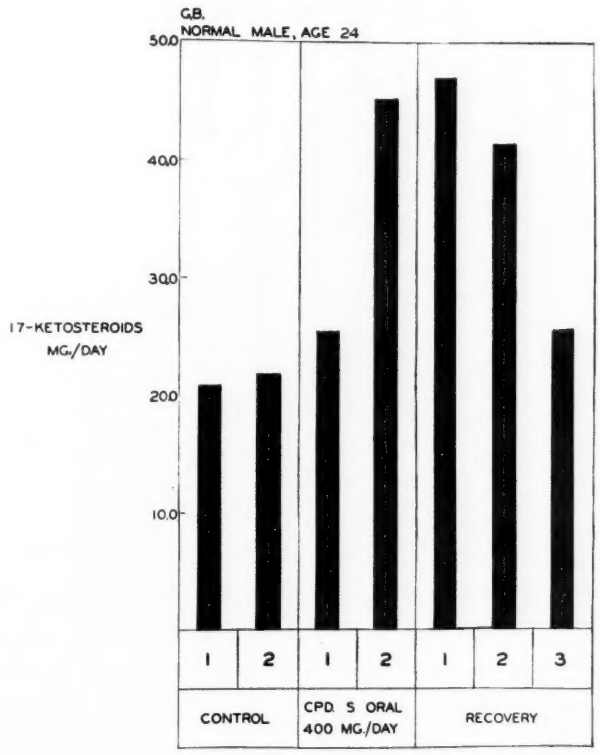

Fig. 8.—Effect of free compound S on urinary excretion of 17-ketosteroids.

Figs. 7, 8, and 9 indicate that oral administration of free compound S to normal subjects (L. W., G. B., and L. B.) produces a rise in excretion of 17-ketosteroids similar to that obtained when compound S acetate is given.

Figs. 10 and 11 show the results obtained from study of the two patients with atrophic hepatic cirrhosis. Each was given 400 mg. per day of compound S acetate orally. On the second day of administration of this steroid to pa-

tient E. J., the excretion of 17-ketosteroids had increased only 12 mg. above his base-line value. The increment in the case of G. B. was 7 mg. These values are to be compared with increases of 37.6 mg. for normal subject G. A. (Fig. 5) and 34.6 mg. for the Addisonian patient F. H. (Fig. 6) on the comparable day.

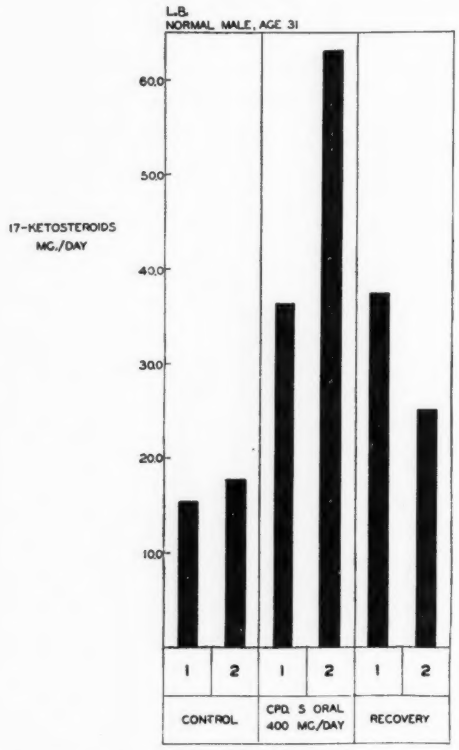

Fig. 9.—Effect of free compound S on urinary excretion of 17-ketosteroids.

Fig. 12 indicates the results obtained on patient N. S. (chronic hepatitis and panhypopituitarism). While on daily maintenance therapy of cortisone acetate, oral administration of 400 mg. of compound S per day increased the excretion of 17-ketosteroids 10.1 mg. above the control value on the second day of administration of the steroid. This compares with increases of 30.9, 25.4, and 46.6 mg. for normal subjects L. W., G. B., and L. B., respectively. After cortisone had been withdrawn for three days, oral administration of compound S for one day caused a rise in excretion of 17-ketosteroids similar to that observed on the first day of administration of compound S in conjunction with cortisone therapy.

Fig. 13 shows the increase in steroidal excretion following the administration of compound S to patient I. R. (improving hepatitis). During oral administration of compound S for four days in March, 1952, there was a minimal

increase in excretion of 17-ketosteroids, but a significant rise in excretion of formaldehydogenic steroids. When these studies were repeated in September, 1952, following considerable improvement in liver function, there was a greater but nevertheless subnormal increase in excretion of 17-ketosteroids. In addition, the increase in excretion of formaldehydogenic steroids was less pronounced than formerly.

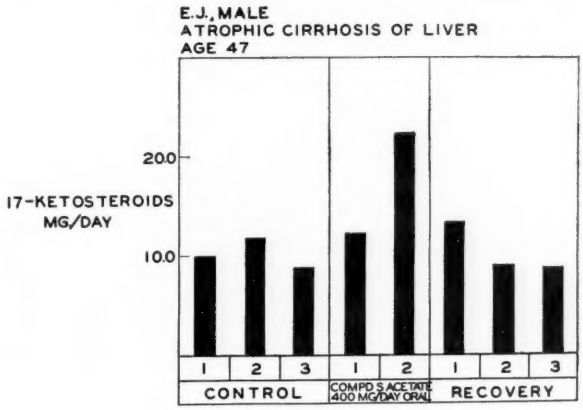

Fig. 10.—Effect of compound S acetate on renal excretion of 17-ketosteroids.

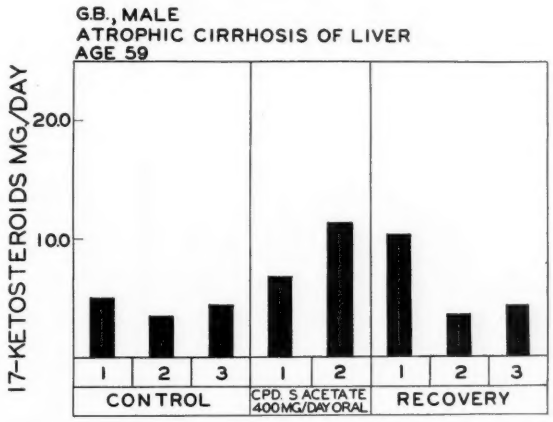

Fig. 11.—Effect of compound S acetate on renal excretion of 17-ketosteroids.

Fig. 14 demonstrates the results obtained in patient E. S. (hemochromatosis). Oral administration of 400 mg. of compound S per day increased the excretion of 17-ketosteroids 7.0 mg. above the control value on the second day of administration of the steroid.

COMMENT

From the results obtained in patients with liver disease it is clear that the normal liver plays a major role in facilitating the conversion of adrenosteroidal

compounds to the 17-keto state. The greater urinary excretion of 17-ketosteroids during oral administration of a given steroid as compared to that observed during intramuscular administration of the same amount of the same compound may be explained by differences in the rate of hepatic conversion when the steroids are given by the two routes. It has been shown[7] that com-

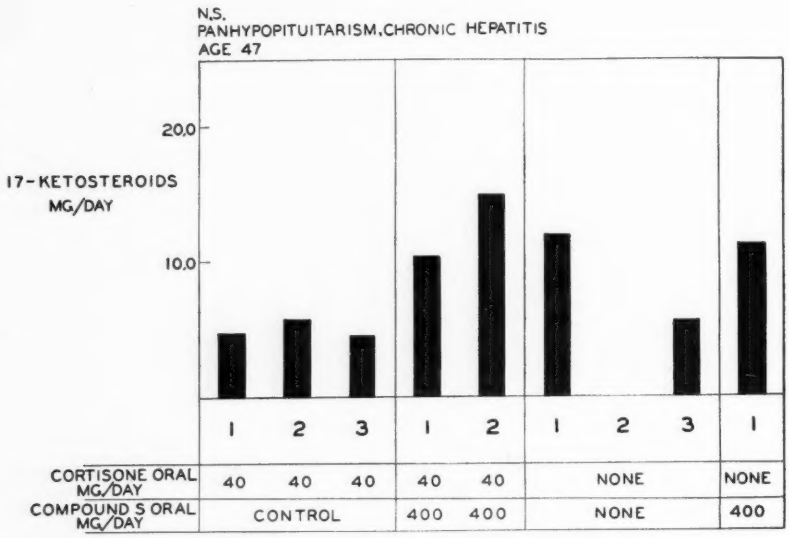

Fig. 12.—Effect of free compound S on urinary excretion of 17-ketosteroids.

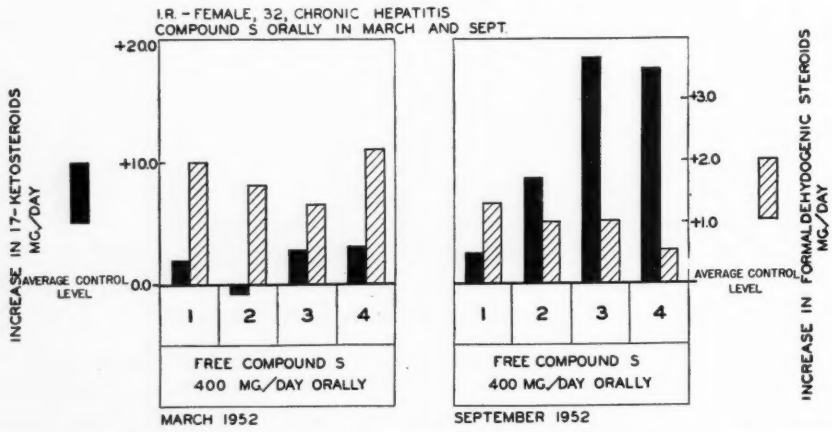

Fig. 13.—Change in urinary steroidal excretion with improvement in liver function.

pound E acetate and compound F and its acetate are absorbed more rapidly when given orally than when administered intramuscularly. Rapid gastrointestinal absorption of a steroid leads to a high concentration of the steroid in the *portal* blood. This probably facilitates its greater hepatic transformation to the 17-keto state. During intramuscular administration of compound F,

compound F acetate, compound E acetate, and compound B (Figs. 1 and 3 and Table II) there occurred either a temporary or sustained decrease in urinary excretion of 17-ketosteroids. This suggests that depression of endogenous precursors of 17-ketosteroids by intramuscular administration of exogenous steroids is greater than the amount of exogenous steroid converted to the 17-keto form. The results obtained suggest (1) that a critical elevation of steroidal concentration in hepatic blood must be obtained before sharply *augmented* conversion to 17-ketosteroids begins and (2) that passage of steroidal compounds through the liver in high concentration is responsible for the greater excretion of 17-ketosteroids when corticosteroids are administered orally. Further evaluation of the factor of concentration of steroids in blood is necessary, and such studies are now in progress.

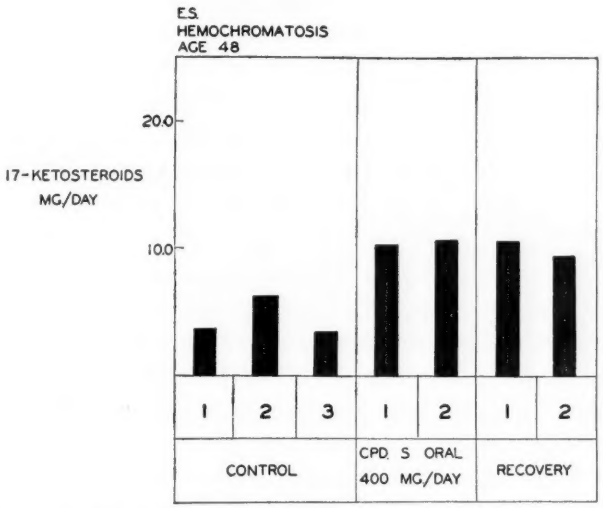

Fig. 14.—Effect of free compound S on urinary excretion of 17-ketosteroids.

The abnormal liver, on the other hand, shows impaired ability to convert adrenosteroids to 17-ketosteroids even at the high portal blood levels obtained following oral administration of a steroid (Figs. 10-14). Butt and co-workers,[8] on the basis of studies involving only the intramuscular route of administration, have also suggested that the damaged liver is incapable of converting cortisone to 17-ketosteroids with the same facility as the normal liver. Deficiency of the abnormal liver in facilitating the conversion of endogenous steroids at normal or high hepatic blood concentrations probably accounts for the frequently observed dissociation of urinary steroidal values in hepatic dysfunction, namely, low values for 17-ketosteroids associated with elevated ones for either reducing steroids or formaldehydogenic steroids.[9-11] Earlier studies[12-22] had also demonstrated that patients with liver disease exhibit subnormal levels of urinary 17-ketosteroids. The greatly impaired conversion of compound S to 17-ketosteroids in patient I. R. (Fig. 10), at a time when liver function was almost normal by conventional tests, suggests that this con-

version rate may prove to be a sensitive test of hepatocellular function. In studies upon normal dogs, Nelson and Harding[23] have concluded, too, that the liver plays a major role in the metabolism of adrenal steroids. These workers found great hepatic arteriovenous differences in the concentration of cortisone following intravenous injection of free cortisone into animals. Significant differences between arterial and venous concentrations of cortisone across other tissues were not observed.

It is of considerable clinical interest in view of our results to ponder from several points of view the meaning of the total urinary 17-ketosteroid excretion. Obviously when this measurement is found to be abnormally high spontaneously, it represents an important observation which at once implies one of several diagnostic possibilities. On the other hand, values which are normal or subnormal may present greater interpretative difficulties.

Low levels of urinary 17-ketosteroids may indicate (a) decreased secretion of adrenocortical and/or testicular hormones, or (b) decreased rate of hepatic transformation of adrenocortical and testicular* hormones at a time when elaboration and release of these precursors of 17-ketosteroids are subnormal, normal, or even supernormal. Similarly, *normal* levels of urinary 17-ketosteroids may be associated, under some circumstances, with decreased hepatic conversion to the 17-keto form of steroids secreted in excess.†

In spontaneous Cushing's syndrome (due to bilateral adrenocortical hyperplasia), even with well-developed clinical and metabolic manifestations, urinary 17-ketosteroids may be within the normal range. Under these circumstances urinary formaldehydogenic steroids may be sufficiently high to be diagnostic. The normal level of urinary 17-ketosteroids may indicate that, although secretion of adrenocortical steroids is excessive, the rate of their hepatic conversion to the 17-keto state is impaired. Fatty infiltration of the liver is a common necropsy finding in Cushing's syndrome.

The increased excretion of reducing or formaldehydogenic steroids and the decreased excretion of 17-ketosteroids in states associated with stress may also be due to decreased hepatic conversion of secreted steroids. Depressed liver function may be a major factor in explaining the low levels of excretion of 17-ketosteroids in patients with malnutrition and cachexia.

The administration of cortisone to patients with congenital adrenal hyperplasia has been shown to decrease urinary 17-ketosteroids. It has been noted that when cortisone is administered orally instead of intramuscularly, larger doses must be given to maintain comparable suppression of excretion of 17-ketosteroids.[25-26] Oral administration of cortisone leads to its greater hepatic conversion to the 17-keto state. To maintain comparable excretion of 17-keto-

*Lloyd and Williams[15] and West et al.[24] have demonstrated the importance of the liver in the metabolism of testosterone. Following intramuscular or intravenous administration of testosterone the concentration of 17-ketosteroids in urine and plasma was lower in patients with liver disease than in normal subjects.

†Since this report was prepared we have studied a patient with severe liver disease who was subjected to the stress of major surgery. No significant change in 17-ketosteroids occurred. But formaldehydogenic steroids rose to the highest levels (31 mg. per day) ever observed in this laboratory.

steroids when cortisone is administered by the two routes, a greater degree of adrenal suppression must be accomplished, when cortisone is given orally. This probably accounts for the larger oral dosage required.

SUMMARY

During administration of compounds F, F acetate, E acetate, B, and S acetate to human subjects, the urinary excretion of 17-ketosteroids is significantly greater during oral administration of each compound than during its intramuscular administration. In contrast, formaldehydogenic steroids increase either equally or to a greater extent when compounds F, E acetate, and B are administered intramuscularly.

Oral administration of compound S and its acetate to patients with liver disease causes a very much smaller increase in excretion of 17-ketosteroids than that observed when the same steroids are given to subjects with normal liver function. This indicates that the liver plays a major role in the transformation of these steroids to 17-ketosteroids. The damaged liver is incapable of performing this function normally.

Passage of adrenal steroids through the liver in high concentration appears to be responsible for the greater excretion of 17-ketosteroids when corticosteroids are administered orally.

The results obtained have important implications with respect to normal hepatic transformation of endogenously produced adrenal steroids. In addition, they probably explain some of the unusual urinary steroidal patterns observed in cases of liver disease and in several other abnormal states.

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MULTIPLE ANTIBODY RESPONSE TO REPEATED TRANSFUSION: DEVELOPMENT OF A HEMOLYTIC ISOIMMUNE ANTIBODY

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INTRODUCTION

THE development of multiple antibodies following transfusion is becoming increasingly recognized.[1, 5] The separate identities of these antibodies are usually demonstrated by specific adsorption with selected blood grouping procedures. The main interest of the case of multiple sensitization herein presented lies in the occurrence of an intragroup isohemolysin, which was responsible for transfusion reactions, and whose existence was not demonstrable by the usual cross-matching procedures.

PERTINENT CLINICAL HISTORY

A 74-year-old white man was admitted to the Los Angeles Veterans Administration Hospital on Oct. 15, 1951, with a constricting lesion of the sigmoid colon and an anemia of 2.8 million red blood cells. Attempted transfusions with whole blood and with washed red cells soon after admission resulted in severe hemolytic reactions after the injection of 150 to 200 ml. of blood. The direct Coombs test was consistently negative. The serum iron was normal (except after a transfusion reaction), and the spleen (at later operation) was small. The patient had received blood at the Veterans Administration Hospital in Van Nuys, California, in 1944 during resection of a carcinoma of the descending colon. No hemolytic reactions were noted at that time. In November, 1948, at the same hospital, two polyps were removed from the splenic flexure of the colon. One of these was benign; the other showed "early malignant degeneration." A transfusion reaction was noted at that time, the exact nature of which is not specified. A month later (same admission) a transurethral prostatic resection was done for benign prostatic hypertrophy. The patient bled with micturition for two weeks thereafter. Two transfusions were again attempted, but on each occasion the administration of 100 to 200 ml. of blood provoked nausea, vomiting, chills, and fever.

In view of this history and of his current status, the patient was referred to the serology laboratory for study. Analysis revealed the presence in the patient's serum of an intragroup isohemolysin, of the Duffy antibody, and of multiple antibodies demonstrable by cross-absorption studies with the conglutinin technique. The first blood which we selected for this patient was given on Dec. 24, 1951, and provoked a hemolytic reaction. This was before the existence of the peculiar isohemolysin had been determined, and brought to a total of three the number of reactions he had sustained on this admission. All had been with bloods selected routinely from blood banks. On Jan. 9, 1952, however, he successfully took 500 ml. of his son's blood. Unfortunately, the patient refused surgery for a number of months. From March 11 to March 13, 1952, he received blood from both his two grandsons and once again from his son, with no clinical or laboratory evidence of hemolysis. His red cell count was now 4 million. At surgery on May 5, 1952, an adenocarcinoma of the sigmoid colon was removed. On pathologic grounds this and the previous neoplasms were considered to be independent carcinomas, rather than metastases. The patient received the blood of one grandson at surgery, under anesthesia, and of the other grandson and of a carefully selected bank blood several days later. None of these provoked a reaction.

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SEROLOGIC ANALYSIS

The patient was in group O, type MP, presumably of the Rh genotype R^2R^2 (cDE/cDE). His red blood cells gave negative reactions with the antisera for the N, S, Kell, Duffy, and Lewis factors. In the initial phase of the study, 300 group O individuals were cross-matched with the patient. Saline cross-matching (major side) was negative in all instances. The conglutinin test was positive in 98 per cent and the indirect Coombs test in 63 per cent of the cross-matching. Only one of the 300 blood specimens seemed compatible with both the last two tests. When given to the patient on Dec. 4, 1951, however, this blood provoked a severe reaction after about 100 ml. had been transfused. The hemolytic nature of the reaction was confirmed by a rise in serum hemoglobin to 68 mg. per cent. The possibility of a bizarre hemolytic antibody was thus called to mind.

Accordingly, equal quantities of a 2 per cent suspension of erythrocytes from this particular donor were set up with serial dilutions of the patient's serum. One-tenth the volume of undiluted guinea-pig complement was added. The mixtures were incubated at 37° C. in a water bath. No hemolysis was noted after two hours, but after twenty-four hours hemolysis was present to a final serum dilution of 1 to 512. The degree of hemolysis decreased in proportion to increasing serum dilutions. No hemolysis was noted in the controls. These consisted of the patient's erythrocytes in his own serum, and of the donor erythrocytes in the serum of another group O individual. During the long pre-operative course of this patient, other bank bloods were found that were compatible with the saline, conglutinin, and indirect Coombs tests, and incompatible with the hemolysin test. The injection of 25 ml. of one of these bloods resulted in a transient rise in the patient's serum hemoglobin.

On the other hand, the patient could tolerate the blood of his son, his two grandsons, and of a carefully selected nonfamilial donor. The role of these transfusions in sustaining him through surgery has been outlined. Adsorption of the patient's serum with excess quantities of tumor tissue failed to alter the titer of the hemolysin to any significant degree. The adsorption was carried out at 37° C. in addition to icebox temperature.

About six months after surgery serum specimens which had been frozen for six and twelve months, together with fresh specimens, were re-examined. The older specimens displayed greater hemolytic activity than the fresh specimens. At this time it was noted that prolonged (twenty-four-hour) incubation was no longer required for the demonstration of hemolysis. Hemolysis was present after one hour and maximal after two hours of incubation, both with the fresh and with the frozen specimens. Furthermore, the hemolytic activity was found to be greater after the serum had been inactivated at 56° C. for thirty minutes. It seems possible that a substance present in the older specimens delayed the sensitization of the red blood cells. This substance may have been destroyed during the prolonged (twenty-four-hour) incubation period of the initial studies, and by storage. The substance was either absent or present in

much diminished titer in the serum taken six months following surgery. Its possible relation to the resected sigmoid carcinoma was not determined.

The final results of the serologic study on this patient are indicated in Table I. The reactions of the patient's serum with the erythrocytes of four members of his family, and with a panel of thirteen selected group O individuals, are outlined. The following points are emphasized:

(1) The Hemolysin.—Titer varied from 1 to 8 to 1 to 128. Complete adsorption of the patient's serum with either cells No. 2 or No. 8 removed the hemolytic activity of the serum for all of the remaining cells of the panel. No correlation existed between the conglutinin and hemolysin tests (see Nos. 3, 7, 10, 11, 13) or between the Coombs and hemolysin tests (see Nos. 10, 12, 13). Further studies (not charted) showed that 64 per cent of a panel of 50 group O individuals gave a positive hemolysin test when set up against the patient's serum. This figure is contrasted with the fact that none of the four members of the patient's family were susceptible to the action of the hemolysin. None of the family members contained any abnormal antibodies in their own serum.

(2) The Duffy Factor.—The reactions with the indirect Coombs test corresponded systematically with the presence or absence of the Duffy factor in the donor erythrocytes. The patient's anti-Duffy titer ranged as high as 1 to 500.

(3) The Conglutinin Test.—Multiple antibodies were present. Even after complete adsorption with four pooled bloods of the panel, agglutination of many of the remaining bloods could be demonstrated.

DISCUSSION

The experiments with selective adsorption of the hemolytic antibody, the two known instances of in vivo hemolysis when that antibody alone was demonstrable, and the failure of the blood of the four family members to show hemolysis with the patient's serum suggest that the hemolysin represented a single and specific intragroup antibody related to the antigenic structure of the red blood cell.

Although intragroup isohemolysis of this general nature is well recognized in cattle,[4] its occurrence in man has infrequently been described.[2] Levine and associates[3] reported a hemolytic isoimmune antibody (anti-Jay) which apparently resulted from isoimmunization to the cells of the patient's own tumor (gastric carcinoma). This was demonstrated by selective adsorption of the antibody from the patient's serum by the tumor tissue. No such adsorption could be demonstrated in the present case.

It may be emphasized that hemolysin tests are not routinely performed in the work-up of hemolytic transfusion reactions; for it is assumed that other tests will pick up all reacting antibodies. We have shown that at least in the present ease such a viewpoint was erroneous, and that only by using hemolysin studies as an actual cross-matching procedure could compatible blood be obtained.

TABLE I. SEROLOGIC REACTIONS OF PATIENT'S SERUM WITH ERYTHROCYTES OF MEMBERS OF HIS FAMILY (ALL GROUP O) AND OF A SELECTED PANEL OF GROUP O DONORS†

DONOR	PROBABLE Rh GENOTYPE	M	N	S	P	KELL	DUFFY	LEWIS	SALINE AGGLUTINATION	CONGLUTININ TEST	CONGLUTININ TEST AFTER ADSORPTION WITH #7	#13	#6	POOLED #2, 3, 5, & 6	COOMBS TEST	HEMOLYSIN TEST	HEMOLYSIN TEST AFTER ADSORPTION WITH #2	#8
Patient	R^2R^2(cDE/cDE)	+	−	−	+	−	−	−							−	−		
Son	R^2R^2(cDE/cDE)	+	−	−	+	−	−	*	−	−					−	−		
Grandson C	R^2r (cDE/cde)	+	−	−	+	−	−	*	−	−					−	−		
Grandson E	R^2r (cDE/cde)	+	−	−	+	−	−	*	−	−					−	−		
Daughter	R^2R^2(cDE/cDE)	+	+	−	+	−	−	*	−	+					−	−		
#1	R^2r (cDE/cde)	+	+	+	−	−	+	−	−	+	−	−	−	−	+	+	−	−
#2	R^2r (cDE/cde)	−	+	−	−	−	+	−	−	+	−	−	+	−	+	+	−	−
#3	R^2r (cDE/cde)	+	+	+	+	−	−	−	−	+	−	−	*	−	−	−	*	*
#4	r r (cde/cde)	+	+	−	−	−	+	−	−	+	−	−	+	−	+	+	−	−
#5	R^2R^2(cDE/cDE)	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	*	*
#6	r r (cde/cde)	+	−	+	−	+	+	+	−	+	−	+	−	−	+	+	−	−
#7	R^1r (CDe/cde)	+	−	−	−	−	−	−	−	+	−	+	+	+	−	−	*	*
#8	R^1R^2(CDe/cDE)	−	+	−	−	−	+	−	−	+	+	+	+	+	+	+	−	−
#9	R^1R^2(CDe/CDe)	−	+	+	−	−	+	−	−	+	−	+	+	+	+	+	−	−
#10	R^1r (CDe/cde)	+	+	+	−	+	+	−	−	+	−	+	+	+	+	−	*	*
#11	R^1r (CDe/cde)	+	+	+	+	−	−	−	−	+	+	−	+	−	−	−	*	*
#12	R^1r (CDe/cde)	−	+	−	+	+	−	−	−	+	−	+	+	+	−	+	−	−
#13	R^1R^2(CDe/cDE)	+	+	−	−	+	+	+	−	+	+	−	+	+	+	−	*	*

1. Conglutinin test. Within the limits of available typing sera, known antibodies to which the patient might have been sensitized were eliminated by the following considerations:

rh^1 (C): adsorption with pooled erythrocytes from which the C factor was absent eliminated the agglutinating power of the patient's serum for blood #11, R^1r (CDe/cde).

hr^2 (e): negative conglutinin test with the two grandsons.

N and Lewis: negative conglutinin test with blood #5.

S: negative conglutinin test with blood #5. Also, adsorption with blood #7, which was S-neg., eliminated the agglutinating power of the patient's serum for six S–pos. bloods (#1, 3, 5. 6. 8, 10).

Kell and Duffy: adsorption with #7 (Kell–neg. and Duffy–neg.) eliminated the agglutinating power of the patient's serum for blood #6 (Kell-pos. and Duffy-pos.).

2. Coombs test. The test was always strongly positive when the Duffy factor was present in the donor cells, and negative when that factor was absent from the donor cells.

3. Hemolysin test. 0.5 ml. of the patient's serum, 0.5 ml. of a 2% suspension of donor erythrocytes, and 0.1 ml. of undiluted guinea pig complement were incubated at 37° C. Two specimens of blood (not charted) were found with negative conglutinin, saline, and Coombs test, but positive hemolysin tests. The hemolysin was therefore a distinct antibody.

*Test not performed.

†The degree of the reactions is not recorded. A + reading indicates a distinct positive reaction, not a one-plus reaction. Readings for the conglutinin and Coombs tests were made both macroscopically and microscopically.

SUMMARY

A case is presented in which the occurrence of a single and specific intragroup hemolytic antibody was noted. This antibody was responsible for hemolytic transfusion reactions. It was active against red blood cells of 64 per cent of fifty donors examined. The patient had apparently been sensitized by previous transfusions. An antibody to the Duffy factor, and certain unidentified antibodies demonstrable by the conglutinin technique were also found. These were distinct from the isohemolysin.

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DIRECT OBSERVATIONS OF INTRAVASCULAR AGGLUTINATION OF RED CELLS IN ACQUIRED AUTOIMMUNE HEMOLYTIC ANEMIA

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THE appearance presented by the blood as it streams through the capillaries in pathologic conditions is often quite different from that seen normally. Thus, the streaming blood of a normal, healthy individual appears to be homogeneous, because the single cells are free, but under pathologic circumstances the red cells often appear to stick to each other, giving a granular or clumpy appearance. In 1920 and 1921 Ploman[1] and Fahraeus[2] described this phenomenon. Later Knisely and his associates[3] made the same observation in a large number of patients and in experimental animals. In the human beings, they studied the flow of blood in the superficial vessels of the conjunctival sclerae by means of a stereoscopic dissecting microscope. Hirschboeck and Woo,[4] using the same technique, studied hospital patients with various diseases and, in agreement with Fahraeus and Ploman, found a close correlation between the clumping of blood cells in vivo and the blood sedimentation rate in vitro. They also observed that anemia appeared to enhance clumping. Zilliacus and co-workers found aggregation of the red cells in the conjunctival vessels of patients with certain gynecologic and obstetric disorders,[5] in allergic conditions,[6] and in rheumatoid arthritis.[7] In allergic disorders, the intravenous injection of heparin was found to reduce intravascular aggregation.[6] Lack et al.[8] observed clumping of the red cells in most patients with arterial hypertension.

Although most investigators agree with Knisely that the distribution of cells in the blood stream in pathologic conditions differs from that seen in normal individuals, divergent opinions with regard to the mechanism and significance of intravascular clumping of red cells have been expressed. Knisely and associates observed a "sticky material" which coated the red cells and caused clumping in the mesenteric vessels of monkeys infected with Knowlesi malaria[9] and after mechanical trauma.[10] Bloch and Powell[11] demonstrated such a coating material on the surface of human red cells by electron microphotographs. Knisely and co-workers used the term "sludging" for the pathologic change in the blood flow.

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In the studies reported here, the blood flow was observed in the conjunctiva by means of the stereoscopic slit lamp microscope. Since it was only possible to make observations of the distribution of the red cells in the streaming blood and not of the viscosity of the blood, we have hesitated to use the term "sludging," which perhaps implies too much. Lutz and his associates,[12, 13] who used the transilluminated hamster cheek pouch for intravascular studies, stated that they had never seen true "sludged blood," although under certain pathologic conditions they found groups of red cells separated by clear plasma in the vessels of the animals. With high magnification, however, they observed some movement of the red cells within the apparently agglutinated or "sludged" groups, and they found no difficulty in separating the undivided cells by simple mechanical means.

The possibility is present that the "granular" or "clumpy" flow is produced in different ways under different pathologic conditions. In some instances there may well be a sticky substance of high viscosity coating the red cells, i.e., a true "sludge." On the other hand, there may be other mechanisms which force the blood cells to pass through the vessels in groups. The purpose of this investigation was not to clarify the mechanism of intravascular red-cell clumping in general. Our particular concern was to determine whether clumping occurs in cases in which the red cells are known to be coated, i.e., in the autoimmune form of acquired hemolytic anemia. In this condition the Coombs test is positive,[14, 15] indicating the presence of human globulin on the surface of the red cells. Zilliacus and Arajarvi[16] noted marked red-cell aggregation in the conjunctival vessels of ten infants with erythroblastosis fetalis, in which the red cells are also coated with globulin and give a positive Coombs test. The aim of the present investigation was to compare the intravascular pattern of the blood in cases of acquired immunohemolytic anemia with that seen in other hematologic cases.

MATERIALS AND METHOD

Fifteen presumably healthy individuals and 53 patients most of whom had a hematologic abnormality were studied. In the main, patients with blood dyscrasias were selected in order to compare the findings in cases of acquired autoimmune hemolytic anemia with those in other types of anemia. Most patients were examined several times, the number of observations varying between one and eleven. Some of the patients with acquired hemolytic anemia were followed for several months. There were, in all, fourteen cases with a positive Coombs test, seven of them "idiopathic," and seven having a more fundamental disease, such as chronic leukemia.

The blood flow in the superficial vessels of the conjunctival sclerae was observed by means of a Bausch and Lomb stereoscopic slit lamp microscope, equipped with 40 mm. objectives and 15× wide field oculars. This combination of lenses gave a magnification of 47×. By means of this apparatus it was always possible to see the blood streaming in the venules, but if the flow was completely normal and smooth, the separate blood cells were not generally visible. If clumping occurred, the blood flow in the vessels was easier to distinguish. In all cases the flow was observed both in venules and in arterioles. It was always much more difficult to distinguish the flowing blood in the arterioles, but in most cases the picture obtained was satisfactory. The capillaries, on the other hand, were barely visible, hence a higher magnification would have been desirable.

The visual impression of such a highly dynamic phenomenon as the blood flow is always more or less subjective. Some kind of measure for comparison of red-cell agglutination, however, was required in cases of acquired hemolytic anemia and other diseases; hence classification in different degrees was resorted to, as previously done by Zilliacus,[5] and by Lack and associates.[8] The flow was considered normal if the blood stream looked completely smooth or if a very fine granularity was observed in only a small minority of the venules only. Grades of clumping were classified as follows: grade 1, fine granularity, i.e., small clumps of red cells in most venules, with inconspicuous clumping or no clumping at all in the arterioles; grade 2, marked agglutination with small and moderate clumps of red cells in all venules and a distinct granularity in most arterioles; grade 3, all cells in the venules sticking together to form clumps of varying size, and at least some of them of considerable size, and marked agglutination in the arterioles. The relationship between clumping in the arterioles and the venules was not always constant, but the classification used seemed adequate to cover all the cases, although it was completely empiric. A detailed protocol was made of each observation.

Our serologic techniques for detecting antibodies in the serum have been described previously by Neber and Dameshek[17] and by Rosenthal and associates.[18] The Coombs test was usually done in test tubes, several antihuman globulin sera being employed, but at times the slide method was used. Titration with an antihuman globulin serum was performed in most cases.

RESULTS

Fourteen patients with acquired autoimmune hemolytic anemia, and having a positive Coombs test, were studied. In all, intravascular aggregation of the red cells was observed, but the degree of clumping varied greatly from case to case. In seven, the disorder was termed "idiopathic." Of those, four were severely ill and anemic; three were chronic cases in good clinical remission when first observed by this technique. Two of the patients in remission showed a very mild degree of intravascular red-cell aggregation (grade 1), one of them so slight that the classification made was 0-1 on two occasions and grade 1 on three occasions. During the time of observation, one of the patients was on a small maintenance dose of cortisone. The third patient, in remission, was observed only once, and a high degree of clumping was revealed (grade 3). He had been splenectomized two years previously, and a short course of corticotropin had been given for three weeks about a year after the operation, when it was shown that splenectomy was without effect. No treatment at all had been given for the past two years. The patient felt perfectly well and was not anemic, but the reticulocyte count was slightly elevated. The Coombs test was strongly positive, but no tests for free antibodies in the serum were made.

Four patients with acquired hemolytic anemia of the idiopathic type were clinically ill and anemic when first observed. The red-cell count was below 3.5 million and the reticulocyte count over 10 per cent. In these four cases, red-cell aggregation in the conjunctival vessels was marked, i.e., grade 2-3 or 3. The patients were then treated with corticotropin and/or cortisone, and, in addition, two were splenectomized. They all responded favorably. The red-cell count increased to normal values in three cases, and the reticulocytes decreased, but the Coombs test remained positive. Intravascular red-

cell aggregation decreased in all cases, but in one case only to grade 1. The remainder still showed a grade 2 clumping at the end of the observation period.

It would be of interest to know whether a correlation existed between the amount of antibodies in the blood and the degree of red-cell agglutination in the vessels. The Coombs test must be considered a qualitative test only, because it is not known whether the so-called "Coombs titer" corresponds to the amount of antibody material on the red-cell surface. Free antibodies were demonstrated in the serum in three cases. The isoagglutinin titer and the microscopic finding, at the beginning and end of the period of observation, are given in Table I.

TABLE I. FINDINGS BEFORE AND AFTER TREATMENT WITH ACTH OR CORTISONE IN FOUR CASES OF ACQUIRED AUTOIMMUNE HEMOLYTIC ANEMIA

		R.B.C.	COOMBS TEST	COOMBS TITER	ISO-AGGL. AT 37° C. SAL.	ALB.	TRYPS.	INTRAVASC. AGGREGATION (GRADES)	
M. F., 70 yr. Female	Before treatment	2.97	++	1:16	-	1:16	1:2	2-3	
	After treatment	3.90	+++	1:16	-	-	1:2	1	
E. S., 63 yr. Male	Before treatment	3.43	+	1:2	-	-	-	2-3	
	After treatment	4.73	+	1:2	-	1:2	1:2	2	
J. J., 77 yr. Male	Before treatment	2.10	+	1:256	-	1:2	1:16	3	cold aggl.
	After treatment	3.63	+		-	1:8	-	2	high titer
B. M., 39 yr. Male	Before treatment	2.95	++++	1:16	1:8	1:8	1:32	2-3	
	After treatment	4.23	++++	1:16	-	1:2	-	2	

As already mentioned, seven patients with an autoimmune hemolytic anemia associated with a more fundamental disease and showing a positive Coombs test were studied in Table II. Four of them had lymphocytic leukemia; three were of the chronic and one of the acute type. Two patients had disseminated lupus erythematosus, and one showed the somewhat confusing picture of pancytopenia, hypoplastic bone marrow, and a positive Coombs test. No patient in this group had demonstrable free antibodies in the serum. All showed some degree of intravascular aggregation of the red cells, varying between grades 1 to 3.

Nine cases of Coombs negative hemolytic anemia were studied. These included three cases of severe Mediterranean anemia, two of hereditary spherocytosis, two of pernicious anemia, and two of unclassified hemolytic anemia. Certainly five, and perhaps all of these, were cases of the "intrinsic" hemolytic anemia. One of the unclassified cases showed a grade 1-2 clumping on first study, but, later, only grade 1 clumping was present. The blood flow of one patient with Mediterranean anemia was completely normal, and clumping of grade 0-1 was found in another. All the remaining six cases in this group showed grade 1 clumping. There was thus some degree of intravascular red-

cell aggregation in most cases in this group, but this was very slight, despite the severity of the anemia in several instances. As an example, one may mention that in a case of pernicious anemia with a red-cell count of 1.53 million per cubic millimeter there was only mild grade 1 intravascular clumping.

The group of neoplastic blood dyscrasias without evidence of acquired hemolytic anemia consisted of 17 cases. The diagnoses included leukemia, lymphosarcoma, Hodgkin's disease, and multiple myeloma. One patient with chronic lymphocytic leukemia revealed almost normal blood flow, estimated as grade 0-1 clumping. In all the other cases there was an unquestionable intravascular aggregation of the red cells. In seven there was grade 3 clumping, and in the remaining cases, grade 2 or grade 1. A very marked degree of clumping was seen in the venules of the patient with multiple myeloma, but clumping in the arteries was slight, consisting only of small clumps.

TABLE II. INTRAVASCULAR CLUMPING IN VARIOUS CONDITIONS

CONDITION	NO. OF CASES	DEGREE OF INTRAVASCULAR AGGREGATION
Autoimmune hemolytic anemia	14	
Relapse Idiopathic	4	2-3
Symptomatic	7	1-3
Remission Idiopathic	3	0-1
Hemolytic anemia .		0-1
Nonimmunologic	9	
Neoplastic blood dyscrasias	17	1-3
Miscellaneous blood dyscrasias	13	1-2
Normal controls	15	0-1

The remaining group of 13 patients included cases of posthemorrhagic anemia, aplastic anemia, and chronic kidney disease (three of each) and single cases of myelofibrosis with myeloid metaplasia of the spleen, chronic granulocytopenia, idiopathic thrombocytopenia, and hypothyroidism. In this group, grade 2 clumping was found in three cases, i.e., in the patient with myelofibrosis, in one patient with aplastic anemia, and in one with posthemorrhagic anemia. A completely normal blood flow was noted in a case of polycystic kidneys with anemia and once in the patient with thrombocytopenia, but upon the second study of the latter case a grade 1-2 clumping was revealed. In all the other patients in this group a mild degree of intravascular aggregation, classified as grade 1, was present.

For control study, 15 members of the hospital personnel were studied. In 11 the blood flow was completely smooth and without agglutination, whereas in 4 a fine granularity in all or some of the conjunctival venules (grade 1) was observed. One of these individuals had a mild respiratory infection and another an asymptomatic allergic condition. Nothing abnormal was found in the remaining two control subjects with grade 1 intravascular aggregation. In this small control material, there was thus no red-cell agglutination in the blood vessels of most healthy people, although two apparently healthy persons revealed a fine granularity of the blood stream.

COMMENT

Erythrocytic antibodies are generally demonstrable in the blood of patients with acquired hemolytic anemia and erythroblastosis fetalis. These are most readily revealed by the Coombs test,[14, 15] i.e., an agglutinin reaction demonstrating a human globulin attached to the surface of the erythrocytes. The Coombs test does not actually prove that the red cells are coated with antibody, although the term "antibody" may be used for the globulin adherent to the cells, since it is probably the direct or indirect cause of red-cell destruction in the so-called autoimmune or immunohemolytic anemias. Also free auto- or isoagglutinins in the serum are found fairly often. Sometimes there are agglutinins active in saline, but more often agglutinins active in albumin solution[17] or antibodies agglutinating only trypsinized red cells.[18] In a few cases it has been possible to demonstrate hemolysins in the serum, at least by particularly sensitive methods,[19] but much more frequently a positive Coombs test or free agglutinins have been found. It may be speculated whether red-cell destruction in autoimmune hemolytic anemia is always the direct effect of hemolysin and complement even in cases where auto- or isohemolysins cannot be directly demonstrated in vitro, or whether pure agglutinins in the serum or on the red-cell surface bring about destruction of erythrocytes in vivo.

According to Knisely and co-workers,[3] and Bloch and Powell,[11] the intravascular erythrocytic aggregation which is found in many different pathologic conditions is caused by a substance adherent to the red cells. This substance is believed not only to cause red-cell agglutination, but also to render the cells susceptible to phagocytosis and, in consequence, to cause red-cell destruction. The coating substance of Knisely, however, must be considered to differ from the globulin which yields a positive Coombs test, because, if it were the same substance, the Coombs test would be positive far more frequently than is the case. In most Coombs-positive cases there is evidence of increased red-cell destruction. It may be assumed that the Coombs-positive globulin (presumably an antibody) causes agglutination in vivo, because (1) it is demonstrable by means of an agglutination test; (2) in many cases free agglutinins in the serum are found simultaneously; (3) autoagglutination of whole blood is often found in immunohemolytic anemia[17, 20]; and (4) eluates of Coombs-positive red blood cells cause red-cell agglutination. The evidence that Knisely's "sludging" factor actually results in red-cell agglutination, phagocytosis, and hemolysis is rather scanty. It is possible that it is concerned more with rouleau formation than with hemagglutination.

It has been proved that pure red-cell agglutinins may cause red-cell destruction when injected into the circulation,[21] as demonstrated by Ham and Castle,[22] and by Dameshek and Miller[23] in experimental animals given intravenous injections of concanavallin A. Producing experimental hemolytic anemia in guinea pigs by the method of Dameshek and Schwartz,[24] Wasastjerna[25] noticed intravascular agglutination, and agglutination in whole blood

taken from the animals after injection of antired-cell serum. Recently Wasastjerna, Dameshek, and Joftes induced immunohemolytic anemia in hamsters by the same method and studied the blood circulation in the transilluminated cheek pouches of the animals. Intravascular agglutination of the red cells was unquestionable, and the phenomenon was recorded on a Kodachrome film.[26]

The investigations now reported revealed intravascular red-cell agglutination in all of the fourteen cases with a positive Coombs test. Agglutination was very slight in the patients who were in good clinical remission, but a high degree of agglutination was found in the clinically active phase of immunohemolytic anemia in man. Patients with a nonimmunologic hemolytic anemia caused by intracorpuscular defects, on the other hand, showed either no, or very slight, agglutination of the red cells in the blood vessels. A high degree of intravascular agglutination of red cells was observed, however, in many severely ill patients with no hemolytic disorder and having a negative Coombs test. This was found particularly in many patients with leukemia or other malignant blood diseases. Similar observations have been made by other investigators in a variety of disease.[3-8]

No qualitative difference was seen between the type of red-cell aggregation found in the Coombs-positive and the Coombs-negative cases showing intravascular agglutination, but it is possible that differences might be visible with improved techniques for observing the circulating blood in man. We know that the life span of the red cells is shortened in cases of chronic leukemia,[27, 28] although to a lesser extent than in many cases of acquired autoimmune hemolytic anemia. Therefore, the fact that intravascular red-cell agglutination is also seen in these cases does not preclude the hypothesis that intravascular red-cell agglutination plays a role in the mechanism for destruction of red cells in immunohemolytic anemia. On the other hand, as intravascular agglutination does not appear to be more marked in acquired hemolytic anemia than in many other severely ill patients, it seems likely that red-cell agglutination is not the only mechanism which destroys red cells in immunohemolytic anemia, but merely one of several mechanisms that shorten the life span of the red cells. It is probable that the erythrocytic clumps suffer more injury in the circulation through mechanical trauma than the free red cells, and that many of them are sequestered in the spleen and other organs.[22]

Another mechanism contributing to the red-cell destruction in immunohemolytic anemias is undoubtedly phagocytosis of the coated red cells, a phenomenon that has been noted in experimental hemolytic anemia,[29] in cases of erythroblastosis fetalis,[30] and in acquired hemolytic anemia.[31] Brandt and associates[32] have demonstrated in vitro phagocytosis of sensitized red cells by macrophages in tissue cultures.

Direct hemolysis as the result of the activity of amboceptor and complement may also be a factor in some cases of immunohemolytic anemia, but, as the hemolysin titer is usually low or absent, this mechanism alone is probably

not responsible for the red-cell destruction. Observations in experimental hemolytic anemia also favor the concept that direct hemolysis is only one phase of the immunohemolytic mechanism in vivo.[25, 33]

SUMMARY

The appearance presented by the blood stream in the superficial vessels of the conjunctival sclerae of 15 healthy persons and of 53 patients with hematologic disease was studied by means of the slit lamp microscope. Intravascular red-cell agglutination was noted in all 14 patients having acquired autoimmune hemolytic anemia. The agglutination was slight to moderate in those patients who were in good clinical remission, and very marked in manifest hemolytic anemia. In cases of hemolytic anemia associated with an intracorpuscular defect, the Coombs test being negative, there was no agglutination or at most fine granularity of the streaming blood, even with severe anemia. All patients suffering from leukemia or other malignant blood diseases revealed a varying degree of intravascular agglutination of red cells. Although the agglutination of red cells coated with antibodies appears to be of pathogenetic importance in the hemolysis of immunohemolytic anemias, it is probable that other mechanisms also contribute to the destruction of red cells in these disorders. It is not clear from these studies as to the relationship of the agglutination phenomenon observed here and the "sludging" phenomenon of Knisely. We have the impression, however, that "sludging," as noted in many conditions, e.g., infections, is more closely related to rouleau formation than to the intravascular agglutination seen in many hemolytic processes.

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COMPARATIVE IMPORTANCE OF SEVERITY AND THERAPEUTIC EFFORT IN DETERMINING THE OUTCOME OF DIABETIC ACIDOSIS AS OBSERVED IN A REPRESENTATIVE GROUP OF PATIENTS*

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THE outcome of a given episode of diabetic acidosis is determined by both the severity of the episode and the treatment received. The relative importance of severity factors as against therapeutic effort in deciding the issue in a given case is often difficult to assess. Comparison of the severity and therapy measures of a group of patients that recovered with those of a group that died of diabetic acidosis might be expected to yield information on this point. Such comparisons were recently made for a representative sample of patients in moderate or severe diabetic acidosis most of whom were treated at the University Hospitals from 1930 to 1948.[1] The therapeutic regimen for these patients was essentially that recommended in the medical texts of the period. There were significant differences between those that lived and those that died for the important severity factors but not for the various treatment measures. It thus appeared, contrary to expectation, that the more severe cases received essentially the same treatment as the milder cases, and the outcome was determined largely by the severity of the episode alone. Before one can accept this conclusion, however, one wants to be sure that the *combined* effect of the various treatment measures was likewise essentially the same for both groups. The present analysis was undertaken to evaluate this point.

METHODS AND MATERIALS

Selection of Variables.—In order to evaluate the various treatment measures during the critical period of therapy the following treatment variables were selected for analysis:

Units of insulin received during first 3 hours.
Units of insulin received during first 6 hours.
Liters of fluid received during first 3 hours.
Liters of fluid received during first 6 hours.
Grams of salt received during first 3 hours.
Grams of salt received during first 6 hours.
Grams of sodium received during first 3 hours.
Grams of sodium received during first 6 hours.
Grams of glucose received during first 3 hours.
Grams of glucose received during first 6 hours.
Grams of alkali received during first 24 hours.

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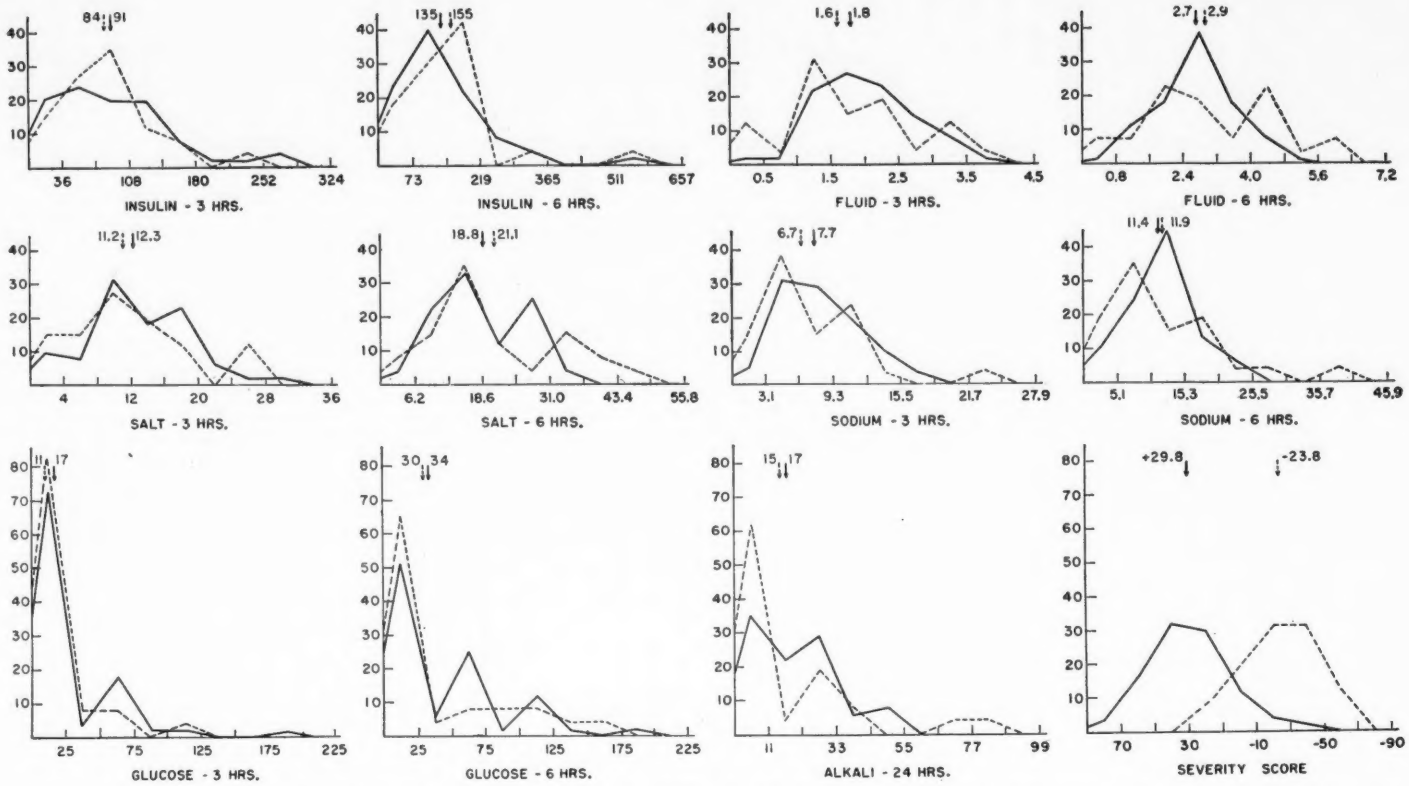

Fig. 1.—Frequency of occurrence of various values of each variable, by group. Ordinates: per cent frequency. Solid line: patients that recovered. Dotted line: patients that died. Numbers and arrows at tops of graphs: mean values.

Certain of these measures are of course highly interrelated; however, this is taken into account in the analysis. In addition to these treatment measures a twelfth variable representing the over-all severity of the acidosis was included for comparison purposes. This measure of severity, the severity score, is an optimal combination of the six most important severity factors, as explained in a recent communication.[2]

Selection of Cases.—Originally, data were available on 124 patients with moderate or severe diabetic acidosis as reported previously.[1] Of this number, 51 patients who recovered and 26 who died in acidosis had complete information recorded for the variables selected for analysis. These patients formed the two groups being compared in the present analysis. The initial data obtained on them are summarized in Table I.

Methods of Analysis.—Our objective is to compare the 51 patients who lived and the 26 who died with respect to each of the variables selected and with respect to an optimal combination of these variables. The process of combining the variables takes into account the ways in which they are interrelated and enables us to give proper weight to each one. The statistical procedures involved in combining the variables have been described in connection with the derivation of the severity score to which reference should be made.[2] The comparative magnitude of the differences between the means of the two groups for each of the treatment and severity variables and for the combinations of variables was determined from analyses of variance in which discrimination ratios were calculated. A *discrimination ratio* is that fraction of the total variation observed among the individuals of both groups that is attributable to average differences between the groups. It enables the various differences between means to be compared with allowances for differences in variability and units of measurement.

TABLE I. MEAN VALUES AND VARIABILITY OF MEASURES SELECTED FOR ANALYSIS

	LIVED (N = 51)			DIED (N = 26)		
VARIABLE	MEAN	S.D.	RANGE	MEAN	S.D.	RANGE
Insulin, 3 hr.	91	63	0-280	84	53	0-250
Insulin, 6 hr.	135	97	0-580	155	111	0-550
Fluids, 3 hr.	1.8	0.7	0-3.5	1.6	0.9	0-3.5
Fluids, 6 hr.	2.7	0.9	0.5-5.0	2.9	1.6	0-6.3
Salt, 3 hr.	12.3	7.0	0-31.5	11.2	7.8	0-27.0
Salt, 6 hr.	18.8	7.9	0-33.0	21.1	13.7	0-49.5
Sodium, 3 hr.	7.7	4.0	0-15.6	6.7	5.2	0-24.5
Sodium, 6 hr.	11.4	4.9	0-24.2	11.9	9.0	0-40.6
Glucose, 3 hr.	17	36	0-200	11	24	0-100
Glucose, 6 hr.	34	44	0-200	30	47	0-150
Alkali, 24 hr.	17	15	0-50	15	23	0-85
Severity score	+29.8	24.2	−31.5 to +88.6	−23.8	21.5	−62.2 to +18.6

RESULTS AND INTERPRETATIONS

Differences Observed Between Group That Recovered and Group That Died With Each Treatment Measure and With the Severity Score.—For each treatment variable, differences between the means of the two groups were calculated and compared with their standard errors to get a measure of the significance of the differences observed (the critical ratio). These critical ratios are listed in Table II along with the corresponding discrimination ratios. It is apparent that of the measures being studied only that representing the severity of the acidosis differentiated between the two groups effectively. Not one of the separate treatment measures shows an appreciable difference between groups. The marked degree of overlapping of the two groups with these measures is illustrated in Fig. 1, which presents the frequency of occurrence of various values of the variables.

Differences Observed Between the Two Groups After Combining the Treatment and Severity Measures to Form a Single Score.—The single score that was derived represents an optimally weighted combination (linear) of eleven treatment measures and six severity factors. The extent to which the two groups are differentiated by it is indicated by its discrimination ratio of 87.7 per cent. The discrimination ratio for the combination of six severity factors (the severity score) alone is 80.0 per cent, and for the combination of eleven treatment measures 7.7 per cent (Table III). The difference between groups for the combination of eleven treatment measures is statistically significant, as shown in Table III, but it adds comparatively little to the separation of groups observed with the severity score alone.

It is evident that in this representative sample of patients therapy was not guided to any appreciable degree by the severity of the episodes, contrary to the intentions of the physicians. Correlations between the severity score and the various therapeutic measures illustrate this further. Had therapy been guided by severity, one would have anticipated moderately high correlations at least. The correlations were in fact low. Thus the correla-

TABLE II. EXTENT AND SIGNIFICANCE OF THE DIFFERENCES BETWEEN GROUPS FOR THE TREATMENT AND SEVERITY VARIABLES

VARIABLE	DISCRIMINATION RATIO (×100)	CRITICAL RATIO
Insulin, 3 hr.	0.3%	0.43
Insulin, 6 hr.	0.8%	0.57
Fluids, 3 hr.	1.2%	0.89
Fluids, 6 hr.	1.1%	0.76
Salt, 3 hr.	0.6%	0.62
Salt, 6 hr.	1.3%	0.85
Sodium, 3 hr.	1.2%	0.88
Sodium, 6 hr.	0.1%	0.24
Glucose, 3 hr.	0.9%	0.92
Glucose, 6 hr.	0.3%	0.43
Alkali, 24 hr.	0.4%	0.45
Severity score	80.0%	--

Discrimination Ratio: Fraction of the total variation among the individuals of both groups that is due to average differences between the groups.
Critical Ratio: Ratio of the difference between means to its standard error.

TABLE III. TEST OF SIGNIFICANCE OF THE CONTRIBUTION OF ELEVEN TREATMENT MEASURES TO THE DIFFERENCES OBSERVED BETWEEN THE TWO GROUPS

VARIATION	df	SUM OF SQUARES	MEAN SQUARE	F
A. Due to combination of treatment and severity variables	17	15.1127		
B. Due to combination of severity factors alone	6	13.7818		
C. Due to combination of treatment measures	11	1.3309	0.1210	3.39
D. Remainder	59	2.1081	0.0357	
Total	76	17.2208		

Discrimination Ratios:

$\frac{A}{Total} = 87.7\%$ $\frac{B}{Total} = 80.0\%$ $\frac{C}{Total} = 7.7\%$

tions between the severity score and the amounts of insulin, fluid, salt, sodium, and glucose given in the first six hours, and the amount of alkali given in the first twenty-four hours were −0.22, −0.23, −0.28, −0.09, 0.11, and 0.10, respectively.

Implications of These Results.—Most physicians take for granted that the more severe cases should receive more vigorous therapy. Analysis of a representative hospital's experience, however, revealed that in actual practice the more severe cases received essentially the same treatment as the milder cases. Why? It seems most likely that the severity of the individual episode was not in general assessed accurately early in the acidosis, and excessive caution characterized therapy during the early critical hours.

An objective estimate of the severity of the acidosis is essential at the time of admission of the patient or very shortly thereafter, since therapy should be guided by severity. Yet without some combination of the severity factors that one must give consideration to, it is difficult to assess the case accurately. As long ago as 1939, Rabinowitch and associates[3] suggested a scheme for combining the severity factors into a single score; their index, however, and a later one by Collen[4] have been used in practice but rarely. The recently described severity score[2] fulfills the same purpose and provides a better measure of the severity of an episode of acidosis. Utilization of such combinations of prognostic factors should lead to more accurate assessments of severity at the time the doctor first sees the patient.

Everyone recognizes that in diabetic acidosis the early insulin is the important insulin. Yet in the parent sample[1] from which the present cases were obtained, less than two-fifths of the insulin given in twenty-four hours was given during the critical first three hours after admission. The same was true for the amounts of fluid, salt, and sodium given. Clearly the need is for more vigorous *early* therapy in the more severe cases. The average amount of insulin given in twenty-four hours to the patients that died (252 units), for example, could more profitably have been given in the first three hours. This need for early vigorous therapy has been stressed repeatedly by Joslin and co-workers[5] who have demonstrated the beneficial consequences of carrying out this policy in practice.

SUMMARY

The relative importance of severity factors and of therapeutic effort in determining the outcome of the acidosis observed in a representative group of diabetic patients was studied by contrasting those that died of acidosis with those that recovered.

There were large differences between the two groups with the important severity factors, but not with the therapeutic measures.

No appreciable correlation was observed between the severity of the episodes and the amount of therapy received. The more severe cases received essentially the same treatment as the milder cases.

These unexpected findings are believed due to inaccurate assessment of the severity of the individual episode at the time of admission, and lack of vigorous early therapy in the more severe cases.

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EFFECT OF DIETS ON THE ANEMIA, AZOTEMIA, AND SURVIVAL OF BILATERALLY NEPHRECTOMIZED RABBITS

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THE demonstration that an anemia,[1] partly hemolytic, develops in the rabbit following bilateral nephrectomy has indicated the need to evaluate the influence of various diets on the level of the blood volume and the hematocrit reading in nephrectomized rabbits. Such was the object of the present study, and at the same time additional information accrued on the influence of diets on the azotemia and survival of this species in the uremia due to bilateral nephrectomy.

METHODS

White or spotted rabbits of either sex, approximately 3 to 4 months of age, weighing 1.4 to 3.7 kg. were used. Six groups of 10 rabbits each and one group of 9 rabbits were studied. The nephrectomies were performed one week apart under ether anesthesia as previously described.[1] The techniques for the T-1824 blood volume determination, the hematocrit reading, and blood urea were those previously used.[1] The plasma chloride concentration was determined by the method of Whitehorn.[2]

Group I consisted of bilaterally nephrectomized rabbits fed a high protein and carbohydrate powder in water in amounts to supply 2 Gm. protein/kg./day, and 21.5 Cal./kg./day. Group II was fasted but given water after the second nephrectomy. Group III was fed a fat emulsion after the nephrectomies in amounts to supply 30 Cal./kg./day. Group IV was fed a 10 per cent glucose solution in water in amounts to supply 2.8 Cal./kg./day. Group V received glucose in water in amounts to supply 30 Cal./kg./day. Group VI consisted of normal rabbits fed fat emulsion in amounts to supply 30 Cal./kg./day. In all of these groups, the animals were given the various diets in suspensions or solutions amounting to 7 ml./kg./day. Group VII consisted of normal rabbits offered regular Purina rabbit chow and water ad libitum during the experimental period.

Diets.—The fat emulsion used contained 40 per cent coconut oil, 20 per cent dextrose with 0.5 per cent soybean phosphatides and 1 per cent polyglycerol esters as stabilizers, prepared in accordance with the instructions given by Gorins, Geyer, Matthews, and Stare.[3] The mixture was emulsified in a Logeman homogenizer and autoclaved as suggested by Shafiroff, Baron, and Roth.[4] The emulsion contained 4.4 calories per milliliter. It did not cause diarrhea in the animals when administered in amounts of 30 Cal./kg./day. The glucose feedings consisted of commercially obtained 10 per cent glucose in water for intravenous use for the low glucose preparation and glucose dissolved in water to supply the high carbohydrate diet. The high protein and carbohydrate powder was Meritene,† which contained 0.325 Gm. protein, 0.544 Gm. carbohydrate and 10 mg. potassium per gram.

The feedings were given once daily by means of gastric intubation with a small polyethylene tube. The tube was readily introduced via the nose while the animal was upright. The animals were fasted for twenty-four hours before the second nephrectomy,

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TABLE I

GROUP	NO. RABBITS	TIME (DAYS)	BODY WT. (KG.)	HEMATOCRIT	BLOOD VOLUME (ML.)	PLASMA VOLUME (ML.)	RBC VOLUME (ML.)	BLOOD UREA (MG./100 ML.)	PLASMA CHLORIDE (MEQ./L.)	SURVIVAL DAYS	CHANGE BLOOD UREA (MG./100 ML.)	CHANGE RBC VOLUME PER CENT
I. Nephrectomies Protein	10	0	2.34 (1.6-2.9)	36.6 (28-40)	200 (150-278)	127 (90-175)	74 (51-103)	65 (38-128)	102 (93-114)	3.8 (3-6)	+237 (157 to 361)	−13.5 (+5 to −25)
		3	2.19 (1.6-2.8)	34.1 (27-41)	186 (154-236)	122 (102-154)	64 (47-82)	302 (195-435)	102 (99-106)			
II. Nephrectomies Fasted	10	0	2.27 (1.6-3.6)	34.5 (18-42)	156 (118-211)	104 (73-172)	52 (39-64)	79 (53-149)	103 (94-114)	5.1 (4-8)	+352 (259 to 457)	−19.2 (−3 to −29)
		4	2.11 (1.6-3.4)	28.9 (18-33)	148 (111-203)	106 (76-165)	42 (35-56)	431 (340-520)	92 (80-106)			
III. Nephrectomies Fat emulsion	10	0	2.32 (1.6-3)	36.4 (29-42)	191 (121-237)	121 (81-153)	70 (40-94)	68 (57-87)	101 (94-109)	5.2 (4-8)	+316 (190 to 470)	−38.6 (−10 to −75)
		4	2.21 (1.5-2.9)	28.5 (20-32)	152 (68-250)	109 (47-177)	43 (21-75)	384 (250-540)	91 (85-106)			
IV. Nephrectomies Low glucose	10	0	2.36 (1.6-3)	35.6 (28-38)	188 (130-262)	122 (83-166)	67 (47-101)	77 (47-137)	99 (95-105)	7.1 (5-14)	+323 (182 to 443)	−22.4 (−2 to −44)
		4	2.24 (1.6-2.8)	30.3 (20-33)	173 (142-213)	121 (96-150)	52 (34-72)	400 (245-490)	91 (82-109)			
V. Nephrectomies High glucose	10	0	1.86 (1.4-2.2)	37.2 (31-42)	182 (134-254)	115 (79-172)	66 (55-82)	68 (47-85)	103 (96-113)	6.7 (5-10)	+221 (116 to 313)	−24.2 (−6.3 to −39.3)
		4	1.76 (1.3-2)	30.2 (25-34)	165 (123-230)	116 (83-166)	50 (42-64)	289 (191-380)	87 (82-100)			
VI. Normal Fat emulsion	10	0	2.58 (2-3.6)	36.4 (32-39)	192 (150-272)	122 (96-172)	72 (52-99)	62 (37-100)	98 (87-106)		+6 (−50 to +58)	−8.4 (−27 to +12)
		4	2.39 (1.8-3.5)	35.1 (27-40)	192 (149-292)	126 (94-191)	66 (45-101)	68 (40-105)	101 (95-109)			
VII. Normal Rabbit chow	9	0	2.40 (2.2-2.6)	35.0 (30-39)	195 (165-214)	127 (100-148)	68 (58-78)	42 (33-56)	100 (94-109)		+1 (−18 to +7)	+1.5 (−9 to +19)
		4	2.47 (2.2-2.7)	34.4 (32-37)	201 (146-263)	132 (93-177)	69 (53-86)	43 (38-50)	101 (95-104)			

0 indicates values taken prior to second nephrectomy.
4 and 3 indicate values taken four and three days after second nephrectomy.
Upper figure indicates mean.
Lower figures in parentheses indicate range.

and feedings were begun on the day of the second nephrectomy. In addition, every other day the animals were given a polyvitamin mixture. The weights were obtained each day before the feeding.

The control blood volume, hematocrit reading, blood urea and plasma chloride concentrations were obtained before the second nephrectomy and the test values were obtained on the fourth day for all groups except Group I, receiving protein, which was studied on the third day. The latter was found necessary in view of the shortened survival of this group. After death, autopsies were performed and no gross hemorrhages or infection were encountered.

RESULTS

The data are summarized in Table I where the mean values and ranges of the various determinations prior to and three and four days after the second nephrectomy are given.

Body Weight.—All groups, with the exception of Group VII (normal rabbits fed rabbit chow), regardless of the caloric intake, lost from 0.1 to 0.2 kg. of weight during the observation period. Even the normal animals fed the fat emulsion (Group VI) lost an average of 0.19 kg. in four days. The most plausible explanation for the weight loss seemed to be the normal evacuation of intestinal contents and their replacement by readily absorbable fluids. Diarrhea did not occur.

Hematocrit Reading.—The mean hematocrit reading was lowered in all nephrectomized animals. The decrement was greatest (average minus 7.9 and 7.0 per cent) in the nephrectomized groups fed the fat emulsion (Group III) and a high glucose diet (Group V). The animals fed a low glucose diet (Group IV) and those fasted (Group II) had decrements amounting to minus 5.3 and 5.6, respectively. The least decrement (minus 2.5 per cent) was observed at three days in the group fed protein (Group I). The normal animals, acting as controls, showed a 1.3 per cent decrement when fed the fat emulsion (Group VI), a 0.6 per cent decrement when given the regular Purina chow (Group VII).

Total Blood Volume, Plasma Volume, and Plasma Chloride Concentration.—The total blood volume was slightly lowered (average 8 to 17 ml.) in all of the nephrectomized groups (Groups I, II, IV, and V) except the group fed the fat emulsion (Group III) which showed a decrement of 39 ml. The plasma volume was either unchanged or slightly lowered in all groups. The plasma chloride concentration was lowered in all nephrectomized groups (Groups II, III, IV, and V) except the one fed protein (Group I). The blood volume, plasma volume and chloride concentration of the two normal groups (Groups VI and VII) were practically unchanged.

RBC Volume.—The red blood cell volume decreased in all nephrectomized groups. The greatest decrement (minus 27 ml.) was observed in the group fed the fat emulsion (Group III). The groups fed glucose (Groups IV and V) and the group that was fasted (Group II) had similar decrements, varying between minus 16 and 10 ml. The fat emulsion appeared to be conducive to anemia as demonstrated by an average decrement of 6 ml. of the RBC volume in the normal group fed fat emulsion (Group VI). Thus, the potentiation of the anemia in the

nephrectomized group fed the fat emulsion may have resulted from this additional influence of this particular diet. The group receiving protein showed a 10 ml. decrement on the third day, which extrapolated in a linear fashion to the fourth day indicated a 13 ml. decrement then. The latter figure is sufficiently close to the glucose and fasted groups (Groups II, IV, and V) as not to appear significant. The normal animals on the regular rabbit diet, handled otherwise in the same manner, remained essentially unchanged, an indication that the anemia was not potentiated by the technique of sampling blood from the heart. Previously it had been shown that manipulations of the kidneys under anesthesia without their removal in ten control rabbits did not cause a decrement in red cell volume.[1]

Blood Urea Concentration.—The initial average blood urea concentration varied between 42 and 79 mg. per 100 ml. Four days after the second nephrectomy the group fed fat emulsion (Group III) showed an increment of 316 mg. per 100 ml., while the group fed a low glucose diet (Group IV) and the fasted group (Group II) showed increments of 323 and 352 mg. per 100 ml., respectively. The group fed protein (Group I) revealed a 237 mg. per 100 ml. increment in 3 days, which projected to 4 days at the same rate would indicate an expected level of 316 mg. per 100 ml. at 4 days. Thus, these four groups of nephrectomized rabbits revealed comparable rates of elevation of the blood urea concentration, with possible exception that a higher degree of azotemia was revealed in Group II. The group fed the high glucose diet (Group V) revealed the least increment in the blood urea concentration (plus 221 mg. per 100 ml.) which was 131 mg. per 100 ml. less than the fasted group (Group II). No changes in the blood urea occurred in the normal groups fed fat emulsion and regular rabbit chow (Groups VI and VII).

Survival Time.—The group fed protein (Group I) had the shortest survival (average 3.8 days, range 3 to 6 days). The fasted group (Group II) and the group receiving the fat emulsion (Group III) had comparable survival periods, for the former an average of 5.1 days (range 4 to 8 days) and for the latter an average of 5.2 days (range 4 to 8 days). The most prolonged survivals were observed in the two glucose groups (Groups IV and V), the low glucose having an average survival of 7.1 days (5 to 14 days) with the high glucose averaging 6.7 days (5 to 10 days). The normal rabbits survived all of the manipulations in an apparently healthy manner.

The observations indicate an adverse influence for the protein diet. The fat emulsion used did not appear to benefit survival over the fasted state. The glucose diets, on the other hand, seemed to prolong the survival period.

COMMENT

The decrement in the blood volume and hematocrit reading in all of the nephrectomized groups indicated the development of an anemia. Previous studies in this laboratory demonstrating a hemolytic mechanism in the genesis of this anemia[1] were supported by the rapidity of the development of the anemia

in the absence of evidence of hemorrhages in life or by post-mortem examination. The anemia occurred despite carbohydrate and fat diets in quantities up to 30 Cal./kg./day. Thus the caloric intake per se, as well as the fasted state, did not appear to alter the anemia. The intake of protein in amounts of 2 Gm./kg./day along with a total of 21.5 Cal./kg./day may have lowered the anemia slightly. The greatest degree of anemia after nephrectomy occurred following the fat emulsion. Since a mild anemia occurred in normal rabbits receiving the fat emulsion, this finding likely resulted partly from the fat diet itself. The tendency of similar preparations to produce anemia has been recorded.[5, 6]

No relationship was established between the anemia and the level of azotemia. No relationship was observed between the level of anemia and the survival. Thus, the group receiving protein had a definite shortening of survival, similar to that reported for the rat and dog and reviewed in general by Masson, Corcoran, and Page[7]; yet the degree of anemia was least pronounced in this group. This effect seemed likely to be due to the potassium content of the diet since the azotemia was proportionately no greater than in the groups fed other diets. At the same time, the survival of the group receiving fat emulsion and fasted was comparable, yet the former group displayed a distinctly more pronounced degree of anemia. The most prolonged survival was noted in the carbohydrate groups, which displayed a level of anemia similar to the fasted group.

Considering the three criteria of anemia, degree of azotemia, and duration of survival, the high glucose group (Group V) yielded the best results, for the survival averaged 6.7 days, the azotemia was markedly less than in the other groups, and the anemia was comparable to the low glucose and fasted groups (Groups IV and II) and distinctly lower than the fat emulsion group (Group III). The marked reduction in azotemia lends support to the theory that certain diets reduce endogenous protein catabolism.

The influence of dietary regimens on experimental uremia has been reviewed and studied additionally by Masson, Corcoran, and Page.[7] The clinical aspects of this question have been considered by Kempner,[8] Borst,[9] Bull and co-workers,[10] and Kolff.[12] Diets used clinically have been composed of carbohydrate and fat and have been low or deficient in electrolytes and protein. The adverse influence of protein on experimental uremia of the type herein described, appears definitely established.[7] The beneficial influence of diets consisting mainly of fats on clinical acute renal failure remains an unsettled question. Renal insufficiency of the rabbit lasting four to fifteen days can be considered comparable in duration to most acute renal failures of man. In this species, with the criteria used, carbohydrate alone was more beneficial for uremia than diets containing fat in the emulsified state. This finding is similar to that of Masson, Corcoran, and Page for the rat. The latter workers considered the diarrhea evolved by the fat emulsion as the cause for the difference noted between fat and carbohydrate diets. Diarrhea did not occur in the herein reported observations.

CONCLUSIONS

The influence of diets of protein, fat, and glucose on the uremia of the rabbit due to bilateral nephrectomy has been evaluated by three criteria: the degree of anemia, the level of azotemia, and the survival time.

The anemia was least in the group receiving protein and in the fasted group and greatest in the group receiving the fat emulsion. The emulsion used caused a slight anemia of normal rabbits. The groups receiving glucose and the group fasted had an intermediate anemia.

For this species, as in others in the literature, a high protein intake shortened the survival during the uremia. The effect was apparently partly due to the potassium content of this diet.

Glucose alone appeared more protective for the uremia than protein, fat, and fasting by the criteria used.

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THE EFFECT OF PAROTIDECTOMY ON BLOOD SUGAR LEVELS IN THE RAT AND MOUSE

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IT HAS been reported by Birnkrant[1] that a marked and rapid hypoglycemia follows total bilateral parotidectomy on rats. Since he found no histologic changes in the liver, pancreas, adrenals, spleen, and testicles, he concluded that the parotid glands must elaborate a substance that acts antagonistically to the action of insulin. In a later group of experiments[2] Birnkrant prepared an extract of the parotid gland, derived three precipitates, and, after injection of each of these precipitates, was able to demonstrate a rise of from 57 per cent to 87 per cent in the blood sugar level. He reported that the blood sugar rise, following the injection of one of the precipitates, showed a remarkable similarity to that of diabetic patients. It was found on autopsy that most of the islets of Langerhans were normal in size, shape, and arrangement, but that there was a definite granular degeneration and disorganization of their normal cell pattern. However, Zimmerman[3] has reported that removal of the parotid glands produces no consistent blood sugar changes in dogs.

The present paper reports the effect of total bilateral parotidectomy on blood sugar levels in the rat and in the mouse.

MATERIALS AND METHODS

Nine adult female Sprague-Dawley rats (litter-mates) were used in this experiment. They weighed approximately 320 grams at the time of operation. Nelson's adaptation of Somogyi's method was used to determine blood sugar levels.[4] About 0.4 ml. of venous blood from the tail of each rat was secured for each determination. The first few drops of blood were discarded before taking the sample. The blood samples were put into tubes previously prepared by drying 0.3 ml. of a 1 per cent solution of sodium fluoride and 0.5 ml. (3 mg.) of heparin. Sugar determinations prior to parotidectomy were made on six of the rats after they had fasted for twenty-four hours, but given water ad libitum. These animals were kept under fasting conditions and until the first blood samples were taken, twelve hours after operation. They were then put on feed for two hours and again fasted until the next blood sample was taken, thirty-six hours after operation. For three more rats, all of the determinations were made while the animals were given food and water ad libitum. When not under fasting conditions they were fed Purina Fox Chow.

The parotid glands were removed after a midline incision was made from above the angle of the jaw down to the superior border of the sternum. The skin was then freed and retracted to either side far enough so that the cartilage of each ear was exposed. To insure

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complete exposure of the parotid glands, some of the overlying fat and fat surrounding the submaxillary glands was also removed. The submaxillary glands were then freed from their posterior attachments and lifted in a craniad direction so as to expose a clear field around the jugular veins. Parotid tissue could be seen extending from around the jugular veins to the ears. In some cases the parotid glands were over one-half inch long. The glands were found to ramify throughout the whole exposed area, from the vicinity of the submaxillary glands to the base of the masseter muscles, and down to the cartilage of the ears. The glands were lobulated and somewhat pinkish in color. All dissection in the region of the neck was done with a small bead of cotton held with a fine forceps so as not to injure the jugular veins or any of their tributaries.

In a second group of experiments, C3H/Jax mice obtained from the Roscoe B. Jackson Memorial Laboratory were used. All the mice were males except for 5 females in the nonoperated control group. Twenty-one mice in all, each weighing approximately 23 grams, were used. The animals were fasted for twenty-four hours before tail blood was collected for analysis. The blood was obtained by amputation of the end of the tail four days following removal of the parotids. The Folin micro method was used to determine the blood sugars in mice.[5] Out of ten mice in which the parotids were removed, only four remained alive, because of excessive bleeding from the neck region. As a control, two groups of C3H/Jax mice were used. In the first group only the submaxillary glands were removed, while in the second one no operative procedure was performed. Blood samples were taken from these animals at the same time and under the same conditions as from the group that had been parotidectomized. The rest of the experimental procedure was the same as that followed for the rats.

RESULTS

The results for the parotidectomized rats are shown in Table I. The blood sugar levels following total bilateral parotidectomy are within the normal range (80-120 mg. per cent). The animals were also autopsied one week after operation to determine whether any parotid tissue had been overlooked. None could be found.

TABLE I. BLOOD SUGAR LEVELS IN THE RAT

RAT NO.	BEFORE PAROTIDECTOMY (MG. PER CENT)	12 HOURS AFTER PAROTIDECTOMY (MG. PER CENT)	36 HOURS AFTER PAROTIDECTOMY (MG. PER CENT)
Fasting Animals			
1	78	96	75
2	94	88	76
3	69	87	87
4	89	93	102
5	83	77	83
6	82	81	93
Nonfasting Animals			
7	114	77	107
8	112	85	112
9	112	94	108

The results for the 3 groups of mice used are shown in Table II. The parotidectomized mice showed blood sugar ranges well within the normal limits. In the two control groups, one from which the submaxillary glands were removed and the other from which blood was taken from nonoperated mice following a

TABLE II. BLOOD SUGAR LEVELS IN THE MOUSE

		CONTROLS			
SEX	PAROTIDECTOMIZED (MG. PER CENT)	SEX	SUBMAXILLARY GLANDS REMOVED (MG. PER CENT)	SEX	NONOPERATED (MG. PER CENT)
♂	118	♂	137	♂	142
♂	136	♂	118	♂	114
♂	126	♂	146	♂	113
♂	101	♂	135	♂	134
		♂	124	♂	110
		♂	115	♀	122
		♂	149	♀	120
				♀	133
				♀	132
				♀	137

twenty-four-hour fast period, the blood sugars again fell within normal limits. No gross changes were seen in any of the animals. Photographs of histologic sections from the pancreas showed no significant changes in the islet cells.

DISCUSSION

The drop in the twelve-hour postoperative levels in the nonfasting rats may be due to the surgical procedure. That this is the most likely explanation is indicated by the approximation of the thirty-six-hour postoperative levels to those obtained prior to operation. There were no clinical signs of hypoglycemia. Had a rapid and severe hypoglycemia developed, one would expect to find the rats listless or in convulsive seizures. All the animals, however, showed normal activity with no convulsions for a period of seven days following removal of the parotids.

Since the sugar levels of mice are far more sensitive to environmental influences than rats, it was found that their normal blood sugar values covered a far greater range and were considerably higher. In general, it can be said that C3H mice exhibit a normal blood sugar range from about 90 mg. per cent to over 170 mg. per cent. This has been confirmed at the Jackson Memorial Laboratory by extensive studies on fasting blood sugars in mice.[6]

SUMMARY

A total bilateral parotidectomy was performed on rats and mice. There were no gross clinical changes noted and no symptoms attributable to hypoglycemia. Blood sugar determinations showed no significant alterations from the normal for each species. From the data presented it does not appear that the parotid glands play any significant role in carbohydrate metabolism.

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THE METABOLIC EFFECTS OF INTRAVENOUS ADMINISTRATION OF CALCIUM

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LITTLE attention has been given to the mineral alterations which result from the intravenous administration of calcium. Albright and Sulkowitch gave calcium by vein to a patient with idiopathic hypoparathyroidism as part of an investigation concerning the mechanism of action of vitamin D.[1] McCance and Widdowson offered evidence that the intravenous administration of calcium and magnesium to normal persons does not provoke increased fecal excretion of these metals.[2] Baylor and his associates studied the metabolic fate of intravenously administered calcium in three subjects, paying particular attention to changes in calcium and phosphorus excretion.[3] Schilling and Lazzlo have evaluated the rate of urinary calcium excretion following its injection as a measure of bone metabolism.[4] More recently Howard and his associates have presented data which indicate that certain physiologic changes which accompany the infusion of calcium may be helpful in the diagnosis of hyperparathyroidism.[5] The present study concerns the application of a standard calcium infusion technique to the investigation of metabolic bone disease.

MATERIALS AND METHODS

Experimental subjects consisted of normal persons and hospital patients with and without metabolic bone disease. A calculated diet containing relatively constant amounts of calcium and phosphorus was given for at least three to five days preceding the test. Twenty-four-hour urine collections were measured for calcium and phosphorus before, during, and following the experimental day. In a few instances meals were given at the usual times, but more often breakfast was withheld until after the infusion; a full day's quantity of food was eaten even though mealtimes were delayed on the experimental day. A water diuresis was instituted and one- or two-hour urine specimens collected, starting at 7 A.M. and continuing until 5 P.M.; these were measured for calcium and phosphorus. In most female subjects an indwelling catheter was placed in the bladder; in the male patients, specimens were collected by voluntary emptying of the bladder. Venous blood for calcium and phosphorus was drawn at successive intervals midway in the urine collection periods. Starting at 11 A.M. calcium gluconate in 500 ml. of sodium chloride was infused over a three-hour period, the total dose of calcium approximating 10 mg./kg. of body weight. In most instances 600 mg. of calcium were given, 100 mg. during the first, 200 mg. during the second, and 300 mg. during the third hour. Collection of urine was continued after completion of the hourly

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study, so as to provide a complete twenty-four-hour specimen on the experimental day, and collection was continued for one or two postinfusion days. Hospital subjects were ordinarily confined to bed during the entire test; normal subjects were ambulatory before and immediately following the three-hour period of calcium administration.

Blood and urinary calcium were measured by the method of Clark and Collip.[6] Phosphorus was determined by the method of Fiske and Subbarow, using Photol reagent rather than aminonaphtholsulfonic acid.[7] All chemical determinations were conducted in duplicate. From these determinations calculation was made of the pattern of serum mineral change, the acute and twenty-four-hour changes in urinary phosphorus excretion and the degree of retention of a given amount of calcium.

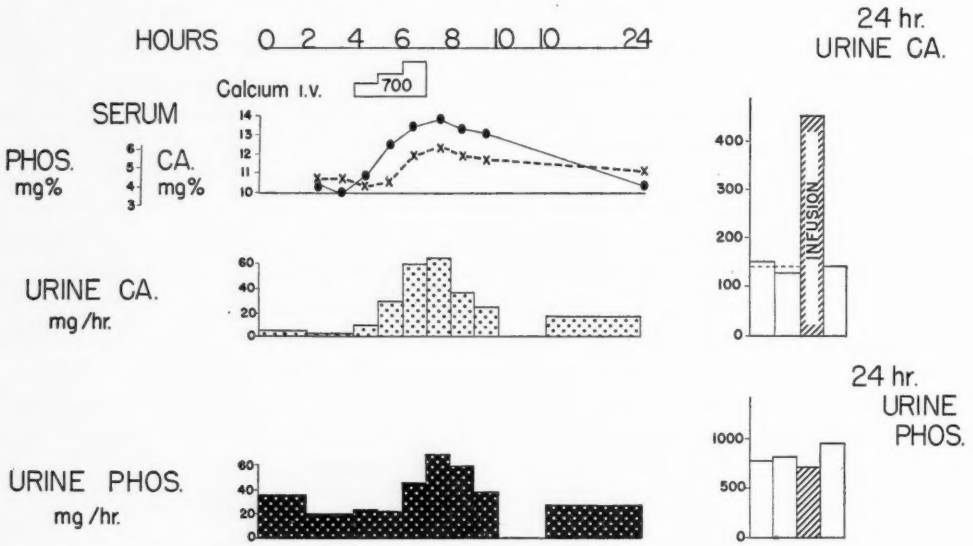

Fig. 1A.—The response to intravenous calcium in a normal subject.

RESULTS

Fig. 1*A* illustrates the response to intravenous calcium in a normal individual. The serum calcium rose from a control level of 10.5 mg. per 100 ml. of blood to a height of 13.8 mg. at the end of the infusion, and returned to normal by the next morning. The serum phosphorus increased 1.8 mg. and remained elevated for several hours. In this instance there was a moderate decrease in urinary phosphorus excretion during the early portion of the infusion but increased excretion was noted thereafter. The twenty-four-hour urinary phosphorus excretion was slightly less on the test day than during the pre- or postinfusion days; in other control subjects phosphorus retention during the experimental day was much more prominent. As the serum calcium rose, urinary calcium excretion increased sharply; this increased rate of excretion was maintained for several hours although at a steadily decreasing rate after termination of the infusion. Approximately 40 per cent of the infused calcium was excreted in the urine, nearly all being rejected on the day of the test.

Fig. 1*B* shows the response in the same individual under the same test conditions to an infusion of isotonic saline without added calcium. The absence

of elevation in the level of serum phosphorus and the relative constancy of urinary phosphorus excretion are in striking contrast to the changes which accompany the infusion of calcium.

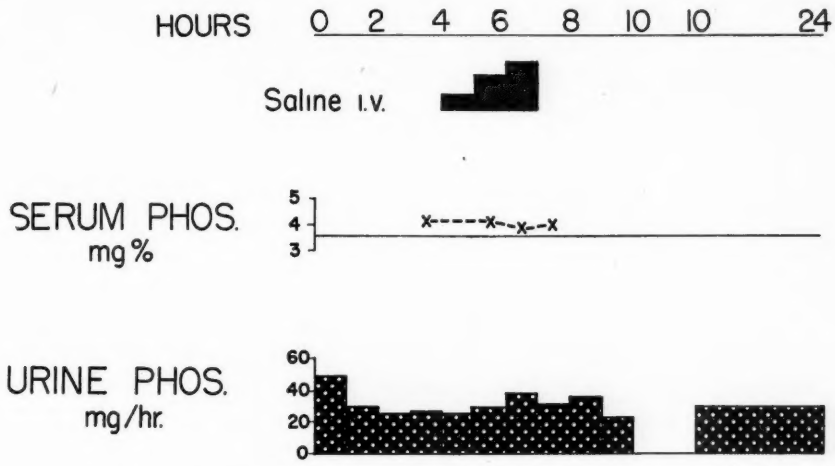

Fig. 1*B*.—The response to isotonic saline in the same individual.

In Fig. 2 the changes in serum phosphorus which accompany calcium infusion are compared percentage wise with the preinfusion values. Significant elevation of serum phosphorus occurred in all control subjects, in osteoporosis and in osteomalacia. The hyperphosphatemia was not related to either the degree of hypercalcemia or the immediate changes in phosphorus excretion. In a patient with hyperparathyroidism due to an adenoma, no change in serum phosphorus accompanied the infusion of calcium on one occasion, but subsequently a slight rise in phosphorus did result from the administration of a larger amount of calcium. In two instances of hypoparathyroidism slight depression of serum phosphorus occurred, despite a significant increase in the level of serum calcium. One patient with renal disease and moderate elevation of serum phosphorus failed to demonstrate further rise during infusion of calcium.

The immediate changes in urinary phosphorus excretion which accompany calcium infusion are shown in Fig. 3, expressed as per cent variation from the rate of excretion during two preinfusion control periods. Results are similar whether change is calculated on the basis of the infusion period alone, or if the infusion and early postinfusion collections are averaged. Although some variability was observed, the majority of the control subjects demonstrated a decrease in phosphorus excretion. In two normal control subjects, who were ambulatory except during the actual infusion, urinary phosphorus excretion increased. In these subjects, however, the rise in serum phosphorus was of the same magnitude as that observed in individuals who showed decreased phosphorus excretion. In both hypoparathyroidism and hyperparathyroidism infusion of calcium was accompanied by increased phosphorus excretion but three instances of osteomalacia demonstrated phosphorus retention during the infusion.

Changes in the twenty-four-hour phosphorus excretion on the experimental day were more constant, all control subjects excreting less in the urine on the test day than during preinfusion days (Fig. 4). In patients with osteoporosis and osteomalacia changes in the twenty-four-hour excretion of phosphorus were comparable to those observed in the control subjects. In a patient with postoperative hypoparathyroidism the infusion of calcium resulted in marked increase of phosphorus excretion, but a case of idiopathic hypoparathyroidism demonstrated significant phosphorus retention. Phosphorus excretion increased slightly when intravenous calcium was given to a patient with hyperparathyroidism due to a tumor.

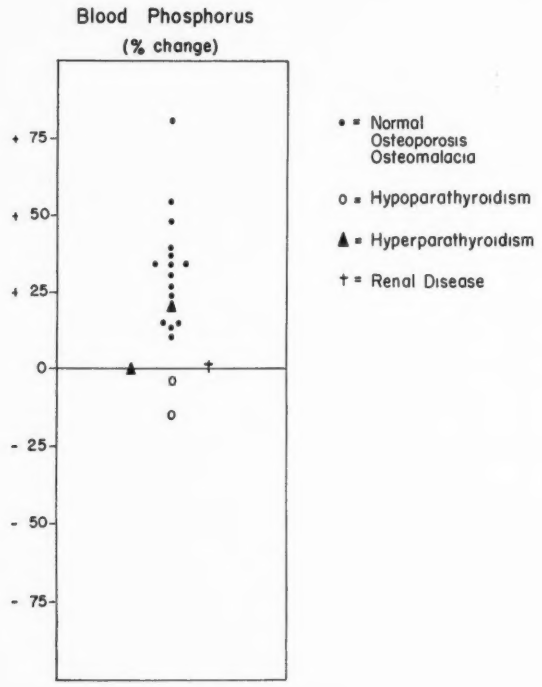

Fig. 2.—Changes in serum phosphorus during infusion of calcium.

Fig. 5 compares the mineral response to intravenous calcium in the two instances of parathyroid insufficiency. The changes in serum calcium and phosphorus and the immediate response of urinary phosphorus excretion were essentially the same in each instance. However, marked decrease in the twenty-four-hour urinary phosphorus excretion occurred in the patient with idiopathic hypoparathyroidism, whereas phosphorus excretion increased in the instance of postoperative parathyroid deficiency. The first patient showed no radiological evidence of demineralization of bones but had persistent elevation of the serum alkaline phosphatase, in the range of 18 to 20 units. However, in this patient calcium retention on the infusion day was no greater than in the patient with

secondary hypoparathyroidism who showed increased phosphorus excretion on the experimental day. In addition, the increment of urinary calcium excretion during the infusion was comparable in these two individuals.

Calcium retention showed considerable variation among the different types of patients. In Fig. 6 this is depicted in terms of percentage of the injected dose, correction being made for the expected urinary calcium excretion taken as the average of two preinfusion twenty-four-hour periods. Calcium retention in normal subjects is of the same magnitude whether comparison is made solely on the basis of excretion during the test day or if values for the infusion and first postinfusion day are averaged. In three instances of untreated postmenopausal osteoporosis, calcium retention on the test day was comparable to that of the control subjects. Significantly greater retention of calcium was observed in

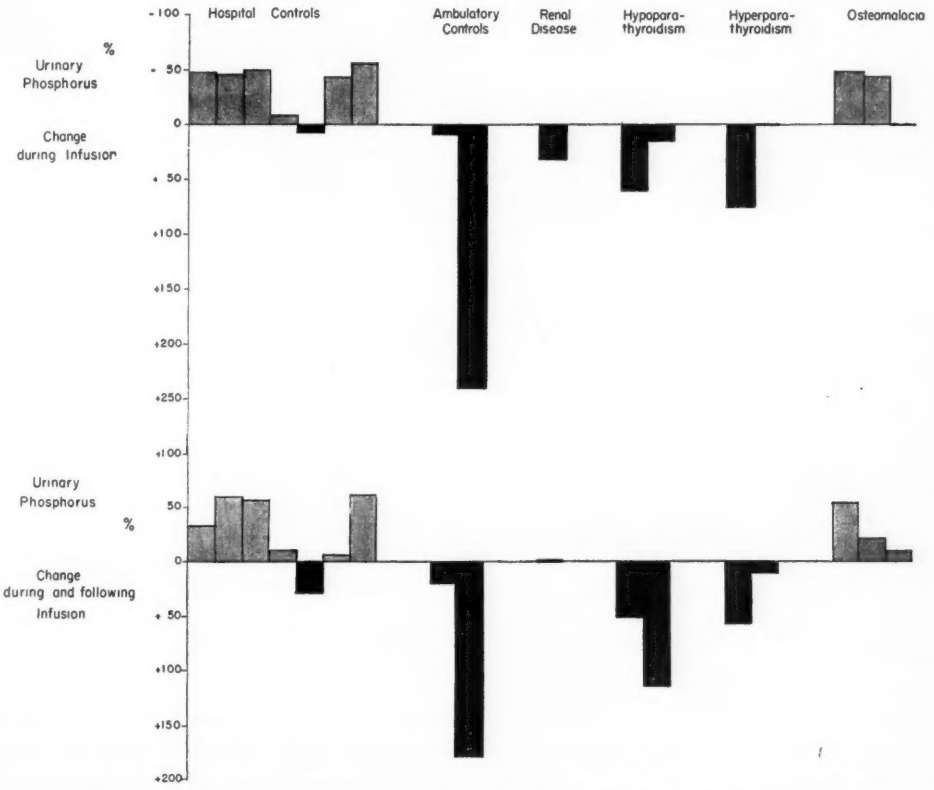

Fig. 3.—Changes in urinary phosphorus excretion during and immediately following the infusion of calcium.

a patient with osteoporosis of Cushing's syndrome and radiological evidence of recalcification who was studied several months after partial adrenalectomy. In two instances of untreated osteomalacia (vitamin D resistant rickets and the DeToni-Fanconi syndrome), retention of calcium exceeded that observed in the control subjects. In a third patient with treated osteomalacia, whose bones showed nearly complete healing despite a very low serum phosphorus, calcium

retention was no greater than that seen in patients without bone disease. As noted previously, both instances of hypoparathyroidism had marked calcium retention despite the dissimilar response of twenty-four-hour urinary phosphorus excretion. A patient with a parathyroid adenoma, who had been ingesting large amounts of milk and alkali, showed normal calcium retention on the infusion day although retention was markedly diminished when postinfusion values were included (a). After he had been on a low calcium diet for three months the test was repeated and, as can be seen in Fig. 6, there was more marked avidity for calcium at this time (b).

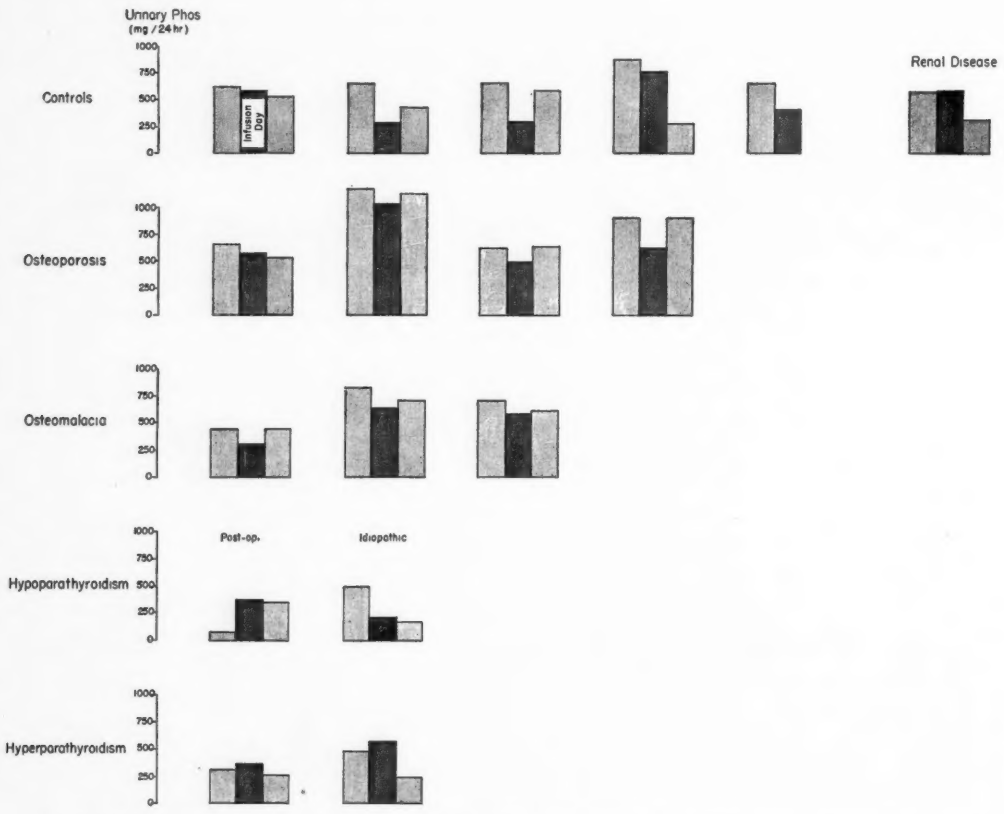

Fig. 4.—Total phosphorus excretion on the experimental day (solid bar) contrasted with excretion before and after the infusion.

DISCUSSION

The most interesting change resulting from the infusion of calcium is the elevation of serum phosphorus. Although frequent mention has been made regarding the reciprocal relationships between serum calcium and phosphorus, rise in serum phosphorus following artificial hypercalcemia has been demonstrated in several experimental studies. In 1924 Salvesen, Hastings, and McIntosh noted that the rise of blood phosphorus after the injection of calcium was a fairly constant phenomena.[8] In 1949 Wolf and Ball observed a rise in

serum phosphorus, associated with a significant decrease in urinary phosphorus excretion following the intravenous administration of calcium chloride or gluconate to dogs.[9] These authors believed their data to be consistent with the idea that calcium loads raise the renal threshold for phosphate but expressed the alternative possibility that an unfilterable colloidal complex of calcium phosphate might be formed. However, studies by Hopkins and Howard show

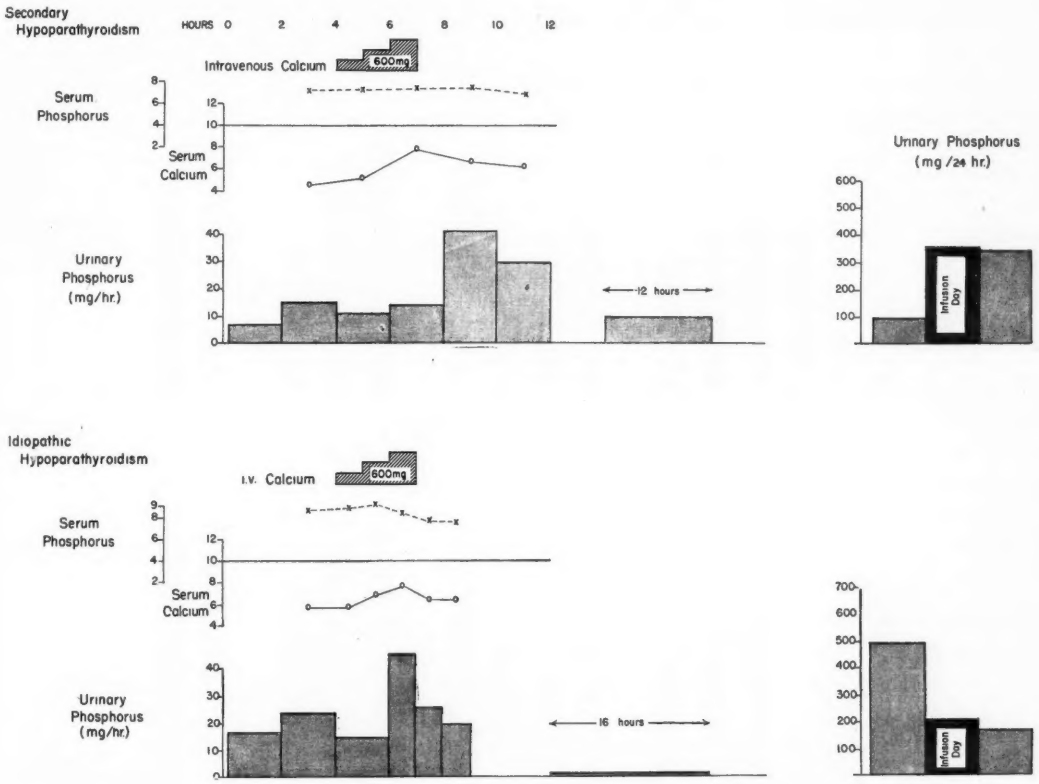

Fig. 5.—The mineral response to calcium infusion in a case of postoperative hypoparathyroidism (top) compared with the response in a patient with idiopathic parathyroid deficiency (bottom).

no in vitro interference with calcium and phosphorus filterability until the serum concentration of these minerals greatly exceeds those levels obtained by the calcium infusion technique described here.[10]

The rise in serum phosphorus cannot be explained on the basis of decreased renal excretion of phosphate. In most instances the rise in blood level was in excess of that possibly attributable to increased tubular reabsorption, even if a purely extracellular distribution compartment for phosphorus is assumed. Furthermore, elevation of serum phosphorus occurred in the two normal subjects who demonstrated increased phosphate excretion during the calcium infusion. In studies on dogs, Hines and Freeman observed elevation of serum phosphorus during induced hypercalcemia which was variable

in degree and which tended to decline as the hypercalcemia diminished.[11] Nephrectomized dogs showed even greater rise in serum phosphorus following the injection of calcium but in animals that were studied forty-eight hours after bilateral ureteral ligation, the serum phosphorus fell initially and then returned to normal levels. Thus the data presented here, as well as those obtained from animal experiments, indicate that the elevation of serum phosphorus which accompanies hypercalcemia is not the result of decreased phosphate excretion.

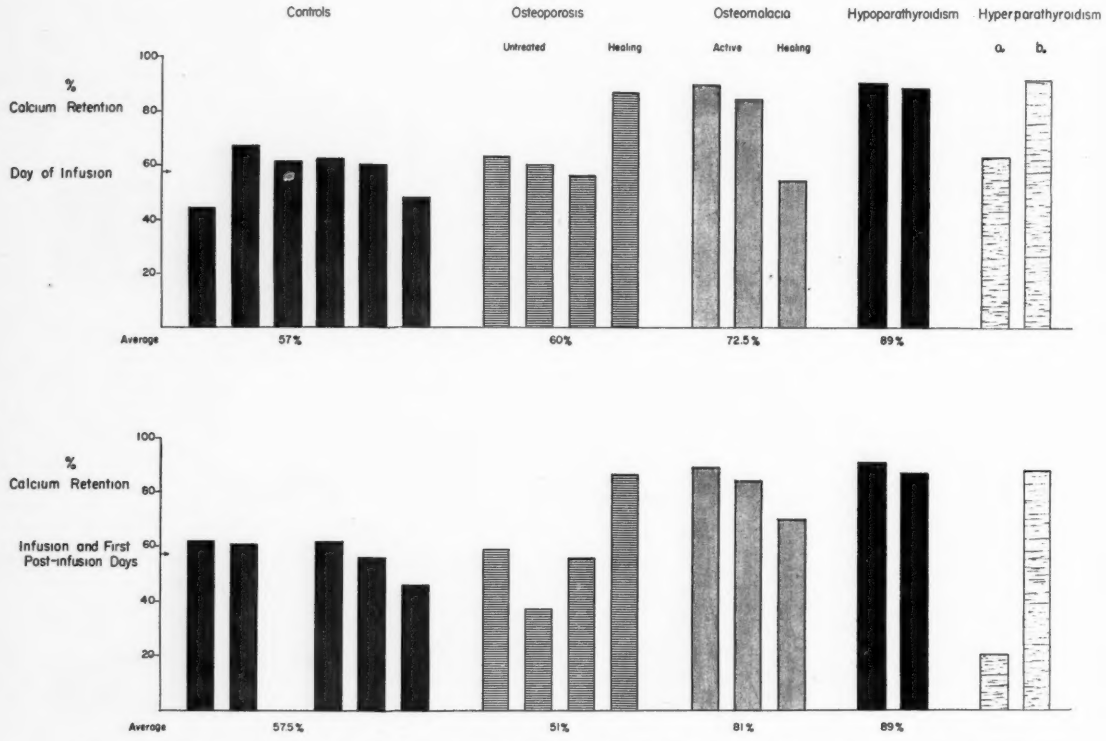

Fig. 6.—The percentage of the infused calcium which is retained in normal subjects and in various types of metabolic bone disease.

Howard has suggested that parathyroid hormone may alter the ratio between organic and inorganic intracellular phosphate and thus, indirectly, affect blood inorganic phosphorus levels.[12] Baylor noted decreased calcium excretion subsequently to the rise which accompanies infusion of calcium, and suggested that this was the result of temporary decrease in the secretion of parathyroid hormone.[3] Although this type of response was not observed in our studies, it is entirely possible that the induction of hypercalcemia may result in temporary suppression of parathyroid gland activity with movement of inorganic phosphate from the cell. Therefore, in the absence of parathyroid tissue, elevation of serum phosphorus should not result from hypercalcemia. This was true in the two patients reported here and to a less constant degree

in the instances of hypoparathyroidism studied by Howard and his associates.[5] The fact that minimal elevation of serum phosphorus occurred in the patient with hyperparathyroidism suggests that secretion of a parathyroid tumor is not controlled by the same physiologic mechanisms which affect normal parathyroid secretion. Thus our data support the concept advanced by Howard that the level of blood calcium may affect parathyroid gland activity, and indicate that phosphate moves from the intracellular space during infusion of calcium. Further investigation of the mechanisms responsible for this interesting change is being conducted.

Decrease in urinary phosphorus excretion during the experimental day appeared to be a constant occurrence in the control subjects. Baylor and his associates noted decreased urinary phosphate excretion following the infusion of calcium and showed that fecal excretion remained unchanged.[3] Howard found either rise or lack of significant decrease in urinary phosphorus in several patients with hyperparathyroidism, these results, together with minimal changes in serum phosphorus, indicating the possible value of the calcium infusion test in the diagnosis of hyperparathyroidism.[5] Our data demonstrate definite rise in twenty-four-hour phosphate excretion in hyperparathyroidism, whereas in normal subjects and in osteomalacia, phosphate excretion decreased. In the patient with renal disease, in whom hypercalcemia failed to induce elevation of serum phosphorus, the renal response was also similar to that seen in hyperparathyroidism. This single observation is mentioned only because of the fact that patients with parathyroid hyperfunction may have renal impairment.

In the patient with secondary hypoparathyroidism there was marked increase in the twenty-four-hour excretion of phosphorus, similar to results reported by Howard. In the instance of idiopathic hypoparathyroidism, however, the opposite result occurred. This striking difference is worthy of comment for, in studies on the physiological effects of vitamin D, Albright and Sulkowitch based their conclusions in part on the observation that in hypoparathyroidism infusion of calcium resulted in marked decrease in phosphate excretion.[1] Howard noted a similar response in an instance of idiopathic hypoparathyroidism, and it is of interest to note that both Howard's patient, and the case reported here showed the rather unusual presence of elevation of alkaline phosphatase. Furthermore, the patient (MGH #4636) with idiopathic parathyroid deficiency studied by Albright is apparently the same individual reported in another communication demonstrating slightly increased alkaline phosphatase activity.[13] It might be suggested that skeletal mineral depletion with consequent need for bone salts was present in all three cases but our patient showed no greater calcium retention than that seen in the instance of secondary hypoparathyroidism. Irrespective of mechanism one is left with the inescapable conclusion that instances of idiopathic hypoparathyroidism with elevation of alkaline phosphatase may react entirely differently from parathyroidectomized patients. This suggests the inadvisability of drawing conclusions regarding mineral physiology from studies on such individuals.

Schilling and Laszlo have used a calcium infusion test to define the state of bone metabolism in a number of skeletal disorders.[4] By measuring the calcium excretion following its intravenous infusion, they demonstrated subnormal rates in patients with a tendency to bone deposition whereas accelerated rates of excretion were found in patients with osteolytic activity. Our studies indicate that a relatively constant amount of the infused calcium is retained in normal persons and similar retention is the rule in untreated osteoporosis. The fact that retention appears slightly lower in the latter group when the infusion and postinfusion data are averaged than when calculation is made on the basis of the experimental day alone, suggests that in osteoporosis, loss of the infused material may continue for a longer period than in normal subjects. In the reparative stage of osteoporosis and in instances of skeletal demineralization significantly greater amounts of calcium are retained. The fact that constant fecal excretion of calcium is maintained following its intravenous administration, as noted by McCance and Widdowson and by Baylor et al, indicates that the portion of calcium not immediately excreted in the urine is retained in the body. The rapid decline of serum calcium to preinfusion levels, and the limitations of the extracellular calcium space indicate that retained calcium must pass into the cellular compartment. The fact that retention of calcium was strikingly increased in hyperparathyroidism after application of a low calcium diet, as compared with the results of the initial study, serves as added proof that the state of bone metabolism can be measured by this technique. The lesser retention of calcium in this patient when rates of excretion during the first postinfusion day are included, indicates that this method of calculation also provides information relative to the activity of bone tissue. These limited data also suggest promise of this technique in ascertaining the effect of treatment designed to encourage bone formation.

SUMMARY

Intravenous administration of calcium results in elevation of serum calcium, persisting for less than twenty-four hours and accompanied by a rise in serum phosphorus. In normal subjects the immediate changes in urinary phosphorus excretion are variable although decreased excretion usually occurs. The elevation of serum phosphorus is apparently unrelated to changes in phosphorus excretion, and appears to be the result of shift of inorganic phosphorus from the cell.

In control subjects less phosphorus is excreted in the urine on the experimental day than during preinfusion periods. In instances of osteomalacia and osteoporosis the change in serum and urinary phosphorus are qualitatively similar to those observed in subjects without bone disease. However, in hyperparathyroidism and secondary parathyroid deficiency, infusion of calcium results in neither elevation of serum phosphorus nor decrease in urinary phosphorus excretion.

Subjects without bone disease retain approximately 60 per cent of the infused calcium, the remainder being rejected on the experimental day. In

instances of osteomalacia, hypoparathyroidism and healing osteoporosis, significantly greater amounts of calcium are retained.

The calcium infusion technique appears to be of value in the study of parathyroid function and for the evaluation of therapy designed to encourage bone healing.

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STUDIES ON THE GLUCURONIC ACID CONTENT OF HUMAN LEUKOCYTES IN HEALTH AND IN DISEASE

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ALTHOUGH the B-glucuronidase activity of serum and leukocytes has been a subject of considerable interest for a number of years, the glucuronic acid content of blood has received only limited attention until recently, and no reports concerning the glucuronic acid content of leukocytes have appeared.

Glucuronic acid is known to participate in various detoxification mechanisms[1] and to form conjugates with the estrogenic[2] and steroid hormones[3] and with epinephrine.[4] It was first quantitatively measured in blood by Mozolowski[5] and by Ratish and Bullowa,[6] and has been observed to show an increase following the ingestion of substances such as the sulfonamides[6] and cyclohexanone.[7] Fishman[8] has also recently shown that ingestion of glucuronic acid orally will produce a rise in glucuronic acid blood levels, and that abnormal blood glucuronic acid tolerance curves may be obtained in the presence of diverse forms of cancer.[8, 9] The serum B-glucuronidase activity was noted to be decreased during the height of the glucuronic acid tolerance curve in both healthy as well as diseased patients. No conclusion could be drawn from the reciprocal effect observed, however.

The purpose of this paper is to report that separated leukocytes contain significant quantities of glucuronic acid in addition to B-glucuronidase, and that variations in their glucuronic acid content occur in leukemia and other hematologic disorders.

METHODS

The separation method for leukocytes has been previously described.[10, 11, 12] Certain modifications were adopted, however, in order to obtain a satisfactory yield of leukocytes with currently available bovine fibrinogen, Fraction I (Armour), as the erythrocyte sedimenting agent. With recent preparations, it has been found necessary to increase the amount of the sedimenting agent in order to obtain maximal yields. After allowing the red cells to sediment, the leukocyte-rich supernatant plasma was aspirated and washed twice with saline, centrifugations being carried out at 1,000 r.p.m. in order to minimize

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platelet contamination. Although a small percentage of erythrocytes remained, this was not felt to be of importance since no glucuronic acid could be demonstrated with repeated determinations on concentrated suspensions of separated red cells.

The leukocytes thus obtained were suspended in approximately 3.0 ml. physiologic saline after the washing procedure, and the cell concentration was adjusted so that it ranged from 50,000 to 80,000 per cubic millimeter. All cell counts were done in triplicate, using Bureau of Standards certified pipettes. The suspension was then centrifuged for ten minutes at 2,500 r.p.m., the supernatant saline aspirated, and replaced to volume with glass-distilled water. After mixing, the resulting hypotonic suspension was allowed to stand for five minutes, and was then homogenized for five minutes in a tight-fitting lucite homogenizer.[13] It was found necessary to use such water homogenates exclusively because of the poor yield of glucuronic acid obtained from whole cells or isotonic homogenates.

Glucuronic acid determinations were carried out by a modification of the method of Fishman.[8] Well-mixed homogenate 2.0 ml. was pipetted slowly into an Erlenmeyer flask containing 6.0 ml. distilled water. This was mixed, and 1.0 ml. each of 10 per cent sodium tungstate and 0.66 N sulfuric acid were added. The volume relationships of all components of the mixture were rigidly maintained at all times, since adequate deproteinization was not obtained otherwise. After ten minutes, this was again mixed and filtered through No. 40 Whatman filter paper. Three ml. aliquots of the filtrate were then pipetted into duplicate glass-stoppered N.P.N. tubes containing the following:

Naphthoresorcinol* 0.2%	2.0 ml.
Concentrated HCl	2.0 ml.

Blank and standard tubes were included, the former containing 3.0 ml. distilled water instead of filtrate, and the latter 20 micrograms glucuronic acid in 3.0 ml. distilled water. The 0.2 per cent naphthoresorcinol was prepared by adding the dry material to an appropriate quantity of distilled water and shaking vigorously for 10 minutes. This effected solution, but filtering was also found to be necessary to remove residual turbidity.

The contents of the boiling tubes were next mixed and placed in a boiling water bath (98° C.) for sixty minutes. Care was taken that the water level of the bath was above the level of liquid in the tubes at all times, and that the tubes did not rest against the sides of the water bath. After incubation, the tubes were immersed in cold water (+5° C.) for ten minutes, and 5.0 ml. 95 per cent alcohol was added to dissolve the blue pigment formed. After agitation of the tubes, water-saturated toluene, 10.5 ml., was added and the colored material vigorously extracted for 1½ minutes. Centrifugation at 2,500 r.p.m. eliminated emulsification, and the toluene extract was transferred to large Coleman cuvettes. Readings were made in a Beckman Model B spectrophotometer at 565 mμ wave length and a final volume of 10.0 ml. The glucuronic acid present was determined by reference to the 20 μg standard, and the results expressed as micrograms of glucuronic acid present per 10^{10} leukocytes. All figures subsequently quoted in this report indicate the number of micrograms of glucuronic acid found per 10^{10} leukocytes.

The use of the naphthoresorcinol test for determining glucuronic acid has been discussed by Diechmann and Dierker,[7] Artz and Osman,[14] and by Fishman.[8] The reaction product obtained by us has the characteristic blue-violet color and light absorption maximum described by these authors. The possibility that other substances may give an identical product has been of considerable concern, but galacturonic acid appears to be the only other substance giving a color with the same characteristics.[8] Galacturonic acid has not been reported a normal constituent of blood, however.[8] Fishman has experienced little or no difficulty with interfering substances in his studies on blood and urine, using toluene as an extracting agent. Previous workers, using ether, have described a brownish pigment[15] which may cause slight interference. Under the conditions of the experiments performed in this study, results showing linear proportionality with tissue concentration were obtained.

*Obtained from Schwartz Laboratories.

Since ascorbic acid is a normal constituent of the leukocyte,[16] and has been referred to as a "hexuronic acid," the possibility that it might cause interference was considered. When 6 μg. of ascorbic acid were added to blank determinations, a pinkish color developed. This gave very little interference spectrophotometrically at 565 mμ wave length, and did not have the characteristic light absorption spectrum.

In our hands, Fishman's method has proved to be rapid and reasonably accurate. Results obtained in duplicate determinations and from day to day have been consistently reproducible. It is recognized, however, that some limiting factors are present, but it is felt that these do not significantly affect the comparative nature of the data presented.

RESULTS

The leukocyte glucuronic acid values found in this study are presented in the form of a scattergram (Fig. 1). Values in normal subjects are compared with those in subjects with neutrophilic leukocytosis, chronic myelocytic leukemia, subacute myelocytic leukemia, lymphocytic leukemia, blastic leukemia, and infectious mononucleosis. Each of these groups will be discussed separately.

Normal Subjects.—These consisted of twenty patients hospitalized for procedures, such as herniorrhaphy, not considered to alter their suitability as normal controls. All were in apparent good health, and in all instances determinations were made prior to any corrective procedure. Leukocyte glucuronic acid values ranged from 2,720 to 5,120, with a mean value of 3,655. The majority of the subjects studied, or 55 per cent, had values between 3,000 and 4,000. Twenty per cent were between 4,000 and 5,000, and, of the remainder, three had values between 2,720 and 3,000, while two were slightly above 5,000. It is apparent that normal leukocytes consistently possess a significant quantity of glucuronic acid which falls within a relatively narrow range for the most part.

Chronic Myelocytic Leukemia.—Eight determinations were done on five patients whose findings satisfied all diagnostic criteria for this condition. Typical differential leukocyte counts demonstrated only a few blasts and promyelocytes, the majority of cells being at the myelocyte, metamyelocyte, and band stages. The range of leukocyte glucuronic acid values was 2,310 to 4,120, the mean value being 2,987, or 668 below the normal mean. All values, as can be seen on Fig. 1, were either low normal or subnormal, except for one. This was a moderately high normal value of 4,120. Two of the five patients studied showed subnormal values and deserve some special comment. Patient H. J. was studied at weekly intervals on three occasions and values of 2,460, 2,999, and 2,310 were obtained. The other patient with a low value, J. F., was found to have 2,570 initially, but two weeks later had a normal value of 3,460. No correlation between the low values obtained and the clinical status could be made in either patient. Likewise, no significant change in the total leukocyte counts was observed, total leukocytes per cubic millimeter in both patients ranging from 106,000 to 135,000 per cubic millimeter during the period of study. Patient J. F. received several x-ray treatments during the period between the low and normal determinations, but no conclusion can be drawn with respect to this.

In addition to the subjects previously mentioned, two additional patients with atypical myelocytic leukemia were studied who were not included with the above group. Their clinical course was relatively more acute, and many atypical cells resembling monocytes were noted on the peripheral blood smear, although the general cell population was similar to that usually seen in chronic myelocytic leukemia. Leukocyte glucuronic acid values were lower than those observed in more typical cases. Patient G. S. showed values of 1,726 and 2,060, respectively, on two occasions. Patient M. H. was studied once only, but was also found to have a low value of 1,487.

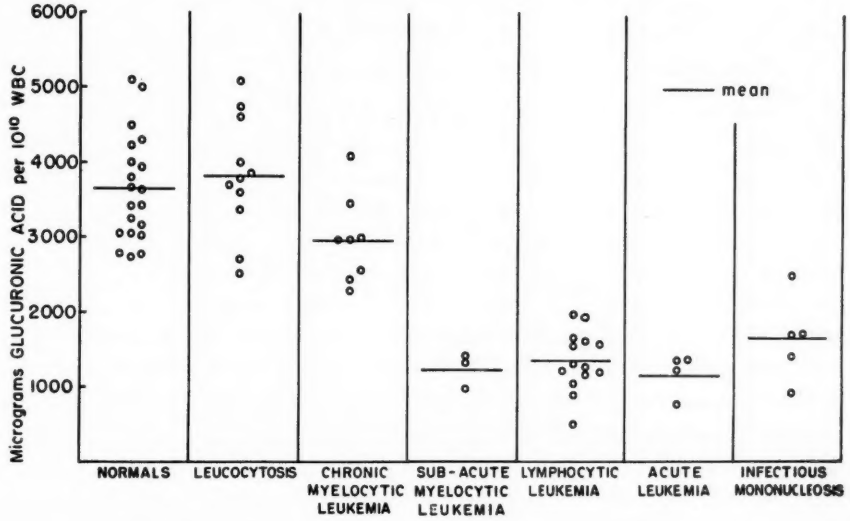

Fig. 1.—Scattergram of glucuronic acid content of leukocytes obtained from normal human subjects and subjects with various disease states. Each dot represents one patient determination. In the chronic myelocytic group, eight determinations were done on five patients, while in subacute myelocytic leukemia, three determinations were done on two patients. In all other instances, only one determination was done on the subject studied. Values are expressed as micrograms glucuronic acid per 10^{10} leukocytes.

Subacute Myelocytic Leukemia.—The two patients studied are represented on Fig. 1. Patient R. M. showed extremely low values of 977 and 1,400, while the value in patient A. G. was 1,360. Thus, low values were observed in both cases, despite marked differences in the hemograms of the two patients. R. M. had a peripheral leukocyte count of approximately 200,000 and 300,000 per cubic millimeter, respectively, at the time of the two determinations. In both instances, the differential formula showed approximately 90 per cent blasts and promyelocytes. In this patient, the cell population was, therefore, predominantly blastic, and the low values observed are comparable to those to be later reported in patients with blastic leukemia and an acute clinical course. Patient A. G., however, showed an entirely different hemogram, even though his clinical course and bone marrow picture were also compatible with a subacute process. The leukocyte count was 28,400 per cubic millimeter, and the peripheral blood smear showed only a few blasts and promyelocytes.

Chronic Lymphocytic Leukemia.—Leukocyte glucuronic acid in subjects with chronic lymphocytic leukemia was also investigated. All patients had typical clinical and hematologic findings. Peripheral leukocyte counts ranged from 18,500 to 230,000 per cubic millimeter. In all cases but one 82 per cent, or more, of the cells were small, mature lymphocytes. In the one exception, 61 per cent of the leukocytes were of this type. The results found in thirteen cases studied are shown in Fig. 1. All values were low, and no overlap was noted with the normals, the range of values obtained being 578 to 1,992, with a mean of 1,401. This was approximately two-fifths the normal mean. Similarly, low values were likewise obtained in a case of lymphosarcoma with the peripheral blood picture of chronic lymphocytic leukemia. The low values observed may be the result of the leukemic process, or may be a function of changes in the predominant cell type. A final conclusion regarding this question cannot be made.

Acute Leukemia.—Three patients with acute blastic leukemia were studied. One, E. M., had 57 per cent blast cells and the glucuronic acid value was 1,350. Another, S. S., had 22 per cent blast cells in association with a high lymphocyte percentage. A value of 1,358 was obtained. The third, H. S., had 89 per cent blast cells, and no demonstrable cells of the myeloid series. His leukocyte glucuronic acid was among the lowest values observed during the study, 783.

These findings indicate that leukocyte glucuronic acid is, in general, very low in the presence of acute blastic leukemia. They also suggest that the blast cell per se is probably very low in glucuronic acid. Only one exception to the uniformly low values obtained in acute leukemia was observed. This occurred in a patient with acute subleukemic leukemia who had only 2 per cent blast cells in conjunction with a neutropenia peripherally, but was found to have a very blastic bone marrow. A normal leukocyte glucuronic acid of 4,010 was found to be present. In this case, of course, the predominant cells analyzed were not themselves blasts.

One patient, M. P., with acute lymphocytic leukemia, was studied during a period of relative remission. The differential leukocyte count showed 90 per cent lymphocytes and 1.5 per cent blast cells at the time of the determination. Previously and subsequently, the peripheral blood leukocytes consisted almost entirely of blast cells and the patient died with typical acute lymphoblastic leukemia. The leukocyte glucuronic acid, 1,230, was similar to the low values obtained in the chronic lymphocytic leukemia group, and in the blastic leukemias.

Neutrophilic Leukocytosis.—Eleven patients with leukocytosis secondary to infection or to miscellaneous causes such as fracture or gastrointestinal hemorrhage were studied. Leukocyte counts varied from 11,225 to 27,750 per cubic millimeter. A moderate to marked shift in the differential formula to the left was present in all cases. The leukocyte glucuronic acid (Fig. 1) showed no characteristic pattern, all values except two falling within normal limits. The range was 2,530 to 5,100, with a mean of 3,829. This varied from the normal mean by only 174. No correlation of leukocyte glucuronic acid values with infection or with neutrophilic leukocytosis was, therefore, possible.

Lymphocytosis.—Five patients with infectious mononucleosis were studied. The leukocyte glucuronic acid values are represented graphically on Fig. 1, and are also listed in the following table:

Patient	*Leukocyte glucuronic acid*	*Lymphocyte % by differential*
J. C.	1,431	90
L. O.	1,714	87
R. T.	914	88
J. R.	1,720	75
G. F.	2,535	49

As can be seen, low glucuronic acid values were found to be present in all patients. Patient G. F. demonstrated the only near-normal value of 2,535. This may possibly be explained by the fact that he had started to improve clinically and hematologically. Two days prior to the determination, 90 per cent lymphocytes, many of which were atypical, had been present, while at the time the determination for glucuronic acid was done the percentage of lymphocytes had dropped to 49 per cent.

The results indicate that low leukocyte glucuronic acid may occur in conditions with lymphocytosis other than the leukemias. Whether this is likewise related to cell type, or to a nonspecific effect, cannot be stated with certainty.

Miscellaneous Conditions.—Two patients with leukemoid reactions secondary to metastatic carcinoma, four patients with Hodgkin's disease, two patients with polycythemia, and five patients with myeloproliferative disease were studied. No characteristic pattern of leukocyte glucuronic acid was observed.

DISCUSSION

The results obtained indicate that human leukocytes contain significant quantities of glucuronic acid, and that this may undergo variation in the disease states studied.

Demonstration of glucuronic acid in leukocytes is dependent upon efficient extraction of the substance from the cell, since no appreciable quantity can be detected with the use of untreated intact cells. Homogenization in a hypotonic solution accomplishes this, presumably through the exertion of both mechanical and osmotic forces upon the cell membrane and intracellular microsomal particles and the mitochondria.[17]

No elevation in glucuronic acid above the normal range was encountered in the patients with disease studied, the only pathologic variation being lowered values. Although low normal to low values were demonstrated for the most part in chronic myelocytic leukemia, this effect was more striking in subacute myelocytic leukemia, and chronic myelocytic leukemia with atypical features. In the latter cases, low values may reflect either the underlying disease process or dilution of the cell population analyzed with cell types naturally poor in glucuronic acid.

Low values were uniformly observed in blastic leukemias, in chronic lymphocytic leukemia, and in infectious mononucleosis. This likewise may be due to a metabolic effect of the disease process upon the predominant cell, the lym-

phocyte or blast, or may be due to an intrinsic deficiency of glucuronic acid in cells of this type. The B-glucuronidase activity of leukocytes in chronic lymphocytic leukemia is also low, and its relation to this condition, in view of the high B-glucuronidase activity present in the lymphocytes of the Malpighian corpuscles of spleen[18] and the low activity of lymph node tissue,[19, 20] has been discussed in a previous report.[10] Whether the simultaneous occurrence of low leukocyte B-glucuronidase activity and low glucuronic acid in chronic lymphocytic leukemia is interrelated, cannot be established with the data presently available. In any event, the low values also obtained in nonleukemic conditions with lymphocytosis indicate that the factor involved is not entirely specific for this type of leukemia.

In view of Fishman's observation that serum B-glucuronidase activity is decreased at the height of blood glucuronic acid curves, the possibility that a similar effect might occur in leukocytes in the presence of certain hematologic disease states was considered. Simultaneous assays for leukocyte B-glucuronidase activity and glucuronic acid content were performed in patients with lymphocytic leukemia, benign lymphocytosis, myelocytic leukemia, nonleukemic neutrophilic leukocytosis, and conditions such as Hodgkin's disease, leukemoid reactions, and myeloproliferative disease. A direct relationship could not be demonstrated, except for the simultaneous occurrence of low values in the presence of lymphocytosis of diverse origin. Glucuronic acid values found in myelocytic leukemia showed no relation to the B-glucuronidase activity. One exceedingly high B-glucuronidase value of 51.4 (normal 22.0 to 35.6)[10] was found in patient A. G. with subacute myelocytic leukemia. The glucuronic acid value was low (1,360). Another patient with subacute myelocytic leukemia, R. M., showed slightly depressed B-glucuronidase activity, but, likewise, had a low glucuronic acid value of 977. A lack of correlation was also evident in the presence of neutrophilic leukocytosis. Two patients with low leukocyte B-glucuronidase activity, associated with severe infection, had normal glucuronic acid values. In the other disease states studied glucuronic acid values varied independently of B-glucuronidase activity.

It is impossible to state with certainty at this time whether or not the glucuronic acid content of leukocytes in the conditions studied is in any way related to their B-glucuronidase activity. The functional implications of B-glucuronidase have been previously reviewed with respect to leukocytes,[10, 21] and other organs,[22, 23, 24] but no unanimity of opinion exists regarding its exact function. Debate has centered primarily around the possible hydrolytic[23, 24] as contrasted to synthetic[22] activity of the enzyme in glucuronide conjugation. Although such metabolic conjugation has been shown to occur in surviving liver slices in vitro,[25, 26, 27] similar activity has not been demonstrated in leukocytes. In spite of this, however, the possibility that the glucuronic acid content of leukocytes reflects cellular detoxification processes, also involving B-glucuronidase, must be considered.

Since certain essential metabolites, such as the estrogens,[2, 28] steroid hormones,[3] and epinephrine,[4] are known to exist in the body as glucuronic acid

conjugates, leukocyte glucuronic acid may bear some relationship to the metabolism of these or similar compounds within the cell. Fishman[8] has suggested that serum B-glucuronidase and blood glucuronic acid may play a role in the metabolism of other body substances containing glucuronic acid, such as hyaluronic acid and chondroitin sulfate. This concept has been lent support recently by the work of Meyer and associates,[29] who have shown that monosaccharides may be produced in vitro from hyaluronic acid previously exposed to hyaluronidase by the hydrolytic activity of B-glucuronidase. The possible relationship of the high B-glucuronidase activity of leukocytes and their significant content of glucuronic acid to this class of compounds is not clear.

Finally, the possibility that leukocyte B-glucuronidase and the metabolism of glucuronic acid by leukocytes may play a role in the general phenomenon of resistance to disease must be considered. This is pertinent in light of the known phagocytic activity of leukocytes with respect to bacteria and the evidence that many bacterial capsular polysaccharides contain glucuronic acid as a portion of their molecular structure.[14, 30]

SUMMARY

Leukocytes contain significant quantities of glucuronic acid in addition to their B-glucuronidase activity.

Leukocyte glucuronic acid is observed to be consistently low in chronic lymphocytic leukemia, and in nonleukemic lymphocytosis.

Blast cells appear to have a very low glucuronic acid content.

In a limited number of cases of chronic myelocytic leukemia, low normal to low values were observed for the most part, and low to very low values were present in the cases studied with certain atypical features and in those with subacute myelocytic leukemia.

No characteristic pattern was found in patients with leukocytosis of infection, Hodgkin's disease, leukemoid reactions, myeloproliferative disease, or polycythemia vera.

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STUDIES OF VITAMIN B_{12} IN SERUM AND URINE FOLLOWING ORAL AND PARENTERAL ADMINISTRATION

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INTRODUCTION

PERNICIOUS anemia, according to current concepts, is the result of deficiency of vitamin B_{12}, brought about by the inability of patients with this disease to absorb the vitamin from dietary sources. This failure of absorption may be due to the absence of "intrinsic factor" from the gastric secretions of these patients.[1] Crystalline vitamin B_{12} is highly effective as a therapeutic agent in pernicious anemia when given parenterally in relatively small doses, but is ineffective when given orally in similar doses unless accompanied by a source of intrinsic factor.[2] However, it has been established that the vitamin may be effective when given alone by mouth, provided that sufficiently large amounts are administered.[3, 4, 5, 6] The mechanism of this apparent absorption of vitamin B_{12} in the postulated absence of the intrinsic factor of Castle is unexplained.

Present estimates of the extent of absorption of vitamin B_{12} from the gastrointestinal tract of normal human subjects and patients with pernicious anemia are based largely upon indirect evidence, such as the evaluation of hematopoietic response when the vitamin is given orally to patients with pernicious anemia,[2] urinary vitamin B_{12} activity following its oral administration,[7] and studies of the excretion of radioactive Co^{60} in the feces following the oral administration of vitamin B_{12} labeled with this isotope.[8]

In order to provide more direct information concerning the absorption, utilization, and excretion of vitamin B_{12} by human subjects, microbiological assay techniques for the determination of vitamin B_{12} activity of serum or urine have been developed in this laboratory.[9, 10] These have been applied to the analysis of serum and urine samples obtained from normal subjects and patients with megaloblastic anemia before and after parenteral and oral administration of the vitamin.

MATERIALS AND METHODS

Normal subjects employed in this study were selected from members of the staff and student body of the Tulane University School of Medicine. Patients with macrocytic anemia were obtained from the wards of the Charity Hospital of Louisiana and from the Tulane

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Nutrition Clinic. All patients were subjected to careful physical and laboratory examination, including roentgenologic studies, gastric analysis with histamine stimulation, and bone marrow aspiration.

During the initial portion of this study, subjects were given a diet in which meat, eggs, fish, milk, and cheese were restricted during the period of observation. However, as subsequent studies revealed that the inclusion of these foods in the diet did not significantly alter vitamin B_{12} activity of either serum or urine, as determined by our methods, subjects employed in the latter portion of the investigation were allowed their usual diet, or, in the case of hospitalized patients, the standard hospital ward diet.

Hemoglobin, hematocrit, and red cell determinations were made three times weekly on venous blood samples obtained without stasis. Daily reticulocyte counts were made on blood smears obtained from finger puncture, stained supravitally with brilliant cresyl blue and counterstained with Wright's stain after drying. Criteria for the evaluation of erythrocyte and reticulocyte responses following the administration of vitamin B_{12} were those proposed by Della Vida[11] and by Isaacs and Friedman[12] for the evaluation of therapy with liver extract. In order to facilitate the comparison of hematopoietic effects obtained with various doses of vitamin B_{12}, and at varying levels of the initial erythrocyte count, the hematologic response observed in each patient has been expressed in terms of the percentage of the optimal response calculated by these methods.

Some of the subjects were studied repeatedly, but in all instances blood counts were either stable or falling at the time of administration of solutions of crystalline vitamin B_{12}. Blood samples for the determination of serum vitamin B_{12} activity were obtained immediately prior to the administration of the vitamin, and, in the majority of instances, at one, four, eight, and twenty-four hours thereafter. Serum was separated after centrifugation and stored in the frozen state until analyzed. All subjects were in the fasting state for at least eight hours when the initial blood sample was taken and, except in subsequently specified instances, food was withheld until after the four-hour sample was obtained. Urine samples were collected for twenty-four-hour periods in bottles containing 10 ml. of glacial acetic acid. Aliquots of the urine were adjusted to pH 6.8 and stored in the frozen state until analyzed for vitamin B_{12} activity.

Microbiological assays of total vitamin B_{12} activity, employing *Lactobacillus leichmannii* (ATCC 4797), were carried out on urine samples according to the method of Thompson and associates,[13] as modified by Register and Sarett,[10] and on blood samples by the procedure of Rosenthal and Sarett.[9] Microbiologically active substances other than vitamin B_{12} have been previously reported to constitute from 0 to 8 per cent of the total urinary vitamin B_{12} activity,[10] and from 0 to 9.4 per cent of the total serum vitamin B_{12} activity[9] in normal subjects. Values given in this report for the total vitamin B_{12} activity of serum and urine of both normal subjects and patients with macrocytic anemia have not been corrected for the presence of this nonspecific activity.

RESULTS

Vitamin B_{12} Activity of Serum and Urine Prior to the Administration of Vitamin B_{12}.—Serum vitamin B_{12} activity of 31 normal subjects ranged from 0.07 to 0.42 mμg. per milliliter in 39 determinations, with a mean value of 0.21 ± 0.013 mμg. per milliliter. The activity of ten samples of serum from six patients with pernicious anemia in relapse was significantly less, ranging from 0.01 to 0.07 mμg. per milliliter with a mean value of 0.04 ± 0.007 mμg. per milliliter (Table I). The total serum vitamin B_{12} activity of two patients with nutritional macrocytic anemia was comparable to that of the pernicious anemia group, ranging from 0.02 to 0.06 mμg. per milliliter. In one patient with polycythemia vera, extremely high serum vitamin B_{12} activity was observed (0.90 and 0.99 mμg. per milliliter). In two patients with secondary polycythemia

and one with a moderate iron-deficiency anemia, serum vitamin B_{12} activity was within the normal range. It thus appears that determination of vitamin B_{12} activity of the serum may be a useful procedure in the diagnosis of macrocytic anemia.

The vitamin B_{12} activity of twenty-four-hour urine samples obtained from patients with pernicious anemia in relapse did not differ significantly from that of the normal group (Table I).

TABLE I. VITAMIN B_{12} ACTIVITY OF SERUM AND URINE OF NORMAL SUBJECTS AND PATIENTS WITH PERNICIOUS ANEMIA IN RELAPSE

GROUP	NO. OF DETERMINATIONS	AVERAGE SERUM VITAMIN B_{12} ACTIVITY (MμG./ML.)	NO. OF DETERMINATIONS	AVERAGE URINE VITAMIN B_{12} ACTIVITY (MμG./DAY)
Normal	39	0.21 ± 0.013* (0.07 – 0.42)†	19	41 ± 6.2* (12 – 135)†
Pernicious anemia	10	0.04 ± 0.007 (0.01 – 0.07)	7	65 ± 13.8 (14 – 121)
Significance		$P < 0.001$		$0.6 > P > 0.5$

*S.E. = Standard error of the mean.
†Figures in parentheses indicate range of values.

Changes in Vitamin B_{12} Activity of the Serum and Urine of Normal Subjects and Patients With Pernicious Anemia Following Intramuscular Administration of the Vitamin.—Changes observed in vitamin B_{12} activity of serum and urine following intramuscular administration of 10, 25, 50, and 100 μg. of the crystalline vitamin to eight normal subjects and of 25 μg. to two patients with pernicious anemia in relapse are shown in Fig. 1. Each curve depicted in the figure represents the observations made in a single subject. Increases in serum vitamin B_{12} activity following injection were prompt. In approximately one hour, maximum values were attained which were roughly proportional to the size of the dose injected. The activity of the serum decreased rapidly within the next three hours, and more slowly for the remainder of a twenty-four-hour period. The curves of vitamin B_{12} activity in the serum of two patients with pernicious anemia who received 25 μg. of the vitamin did not differ significantly from those of two normal subjects who received the same dose, despite the fact that essentially no vitamin B_{12} activity was demonstrable in the serum of either patient prior to the injection. In all subjects, vitamin B_{12} activity of the serum twenty-four hours after the injection was greater than that found before the administration of the vitamin; in the two patients with pernicious anemia, serum activity had decreased to a greater extent than in the normal subjects, but values remained within the normal range.

Vitamin B_{12} activity of twenty-four-hour urine samples following intramuscular injection of the vitamin increased roughly in proportion to the size of the dose administered (Fig. 1). There was considerable variation between individual subjects. Increases in urinary vitamin B_{12} activity of the two patients with pernicious anemia who received 25 μg. of the vitamin were somewhat less than those observed in the two normal subjects receiving the same dose.

The hematologic response to the intramuscular injection of 25 μg. of vitamin B_{12} in the two patients with pernicious anemia was suboptimal in each case (Table II).

TABLE II. SERUM VITAMIN B_{12} ACTIVITY AND HEMATOLOGIC RESPONSE FOLLOWING 25 μG. OF VITAMIN B_{12} INTRAMUSCULARLY IN PATIENTS WITH PERNICIOUS ANEMIA

PATIENT	SERUM VITAMIN B_{12} ACTIVITY (MμG./ML.) INITIAL	MAXIMUM	INITIAL ERYTHROCYTE COUNT (MILLIONS/ C.MM.)	RETICULOCYTE RESPONSE* (PER CENT)	ERYTHROCYTE RESPONSE IN 2 WEEKS* (PER CENT)
E. A.	0.01	0.84	3.40	357	24.6
C. P.	0.00	1.33	2.93	84	52.7

*Reticulocyte and erythrocyte responses are expressed in terms of percentage of the optimal response calculated from the formulas of Isaacs and Friedman[12] and Della Vida.[11]

Changes in Vitamin B_{12} Activity of Serum and Urine of Normal Subjects and Patients With Megaloblastic Anemia Following Oral Administration of the Vitamin.—Vitamin B_{12} was administered orally to ten normal subjects in doses ranging from 500 to 3,000 μg., and to eight patients with megaloblastic anemia in doses of 50 to 3,000 μg.

Following the oral administration of 50 μg. of vitamin B_{12} in solution, one patient with pernicious anemia (R. R.) showed no change in serum vitamin B_{12} activity and no hematopoietic effect. The administration of the same amount of the vitamin with 225 ml. of unneutralized normal human gastric juice was likewise without effect upon serum vitamin B_{12} activity. Although an optimum reticulocyte response followed, no increase in the erythrocyte count was observed (Table III).

TABLE III. SERUM VITAMIN B_{12} ACTIVITY AND HEMATOLOGIC RESPONSE FOLLOWING ORAL ADMINISTRATION OF VITAMIN B_{12} IN PATIENTS WITH PERNICIOUS ANEMIA

PATIENT	DATE	DOSE (μG.)	SERUM VITAMIN B_{12} ACTIVITY (MμG./ML.) INITIAL	MAXIMUM	INITIAL ERYTHROCYTE COUNT (MILLIONS/ C.MM.)	RETICULOCYTE RESPONSE* (PER CENT)	ERYTHROCYTE RESPONSE IN 2 WEEKS* (PER CENT)
R. R.	11/28/52	50	0.03	0.05	2.90	0	0
	12/11/52	50 + 225 ml. gastric juice	0.04	0.06	2.86	112	0
C. P.	3/10/52	500	0.01	0.05	2.17	30	32
	5/14/52	50 × 10	0.04	0.23	3.72	--	101
	2/23/53	500	0.13	0.20	3.74	--	328
E. A.	2/ 5/52	500	0.07	0.32	2.87	131	36
	10/20/52	1,000	0.01	0.85	1.96	50	49

*See footnote, Table II.

When 500 μg. of vitamin B_{12} was given orally to two normal subjects, only minor changes in serum vitamin B_{12} activity occurred within periods of 12 and 16 hours (Fig. 2).

When a similar dose was given to two patients with pernicious anemia in relapse, practically no change in serum vitamin B_{12} activity was noted in one (C. P.), a definite increase in the other (E. A.) (Fig. 2). Hematologic response

followed but was suboptimal in each instance (Table III). Another oral dose of 500 μg. was given to patient C. P. eleven months later, at a time when she was beginning to relapse following discontinuation of parenteral therapy. A slight increase in serum vitamin B_{12} activity and a satisfactory erythrocyte response followed (Table III, 2/23/53).

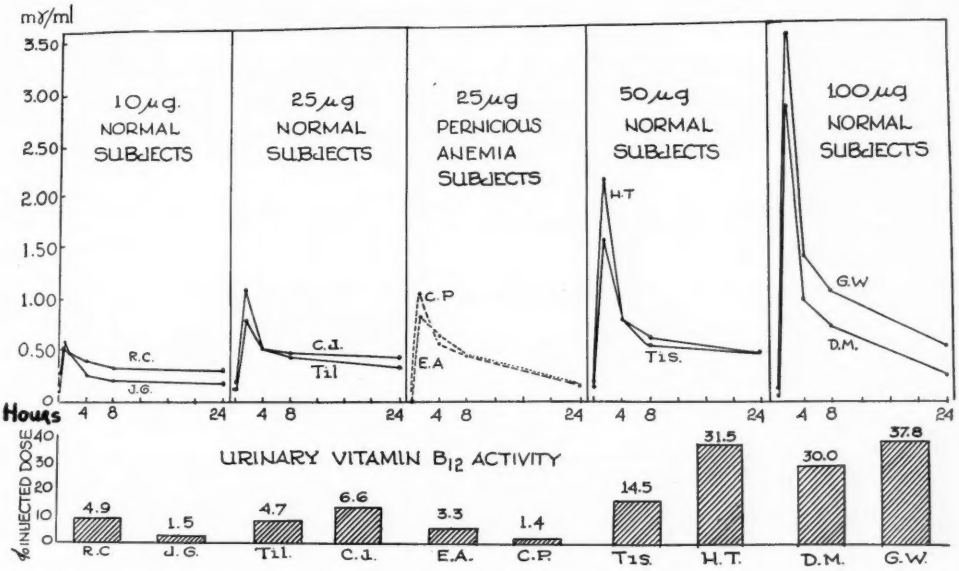

Fig. 1.—Vitamin B_{12} activity of serum and urine of normal subjects and patients with pernicious anemia following intramuscular administration of the vitamin.

When 500 μg. of vitamin B_{12} was given orally in ten doses of 50 μg. each at hourly intervals, a slight increase in serum vitamin B_{12} activity was observed in one normal subject and in one patient with pernicious anemia (Fig. 3). An optimal erythrocyte response occurred in the pernicious anemia patient (Table III, 5/14/52).

Urinary vitamin B_{12} activity following oral administration of 50 to 500 μg. of vitamin B_{12} showed only minor changes in relation to basal excretion. This was true for both normal subjects and patients with pernicious anemia.

After oral administration of 1,000 μg. of vitamin B_{12}, slight increases in serum vitamin B_{12} activity were observed in two normal subjects. One patient with pernicious anemia (E. A.) attained a much more marked effect (Fig. 4). This was followed by a suboptimal hematopoietic response (Table III, 10/20/52). The maximum level of serum vitamin B_{12} activity observed in this instance was essentially identical with that previously attained in the same patient after the intramuscular injection of 25 μg. of the vitamin (Fig. 1). However, urinary vitamin B_{12} increased 883 mμg. following the parenteral dose and only 43 mμg. following the oral dose. In the two normal subjects who received 1,000 μg. orally, urinary excretion increased 20 to 30 mμg., changes of doubtful significance.

Vitamin B_{12} activity of serum and urine following the oral administration of 3,000 μg. of the vitamin was measured in 6 normal subjects and 6 patients with macrocytic anemia. A definite increase in serum vitamin B_{12} activity was observed in 5 of the 6 normal subjects. The increase was less prompt but

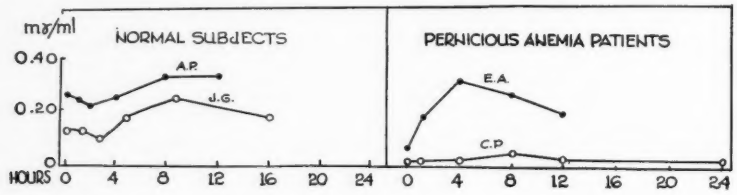

Fig. 2.—Serum vitamin B_{12} activity of normal subjects and patients with pernicious anemia following oral administration of 500 μg. of the vitamin.

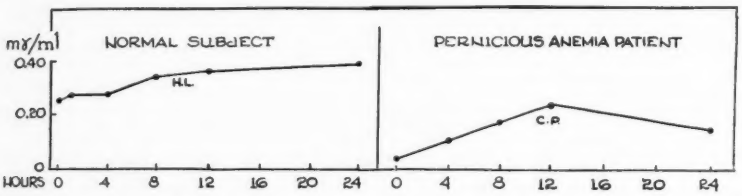

Fig. 3.—Serum vitamin B_{12} activity of a normal subject and a patient with pernicious anemia, following oral administration of the vitamin in ten doses of 50 μg. each at hourly intervals.

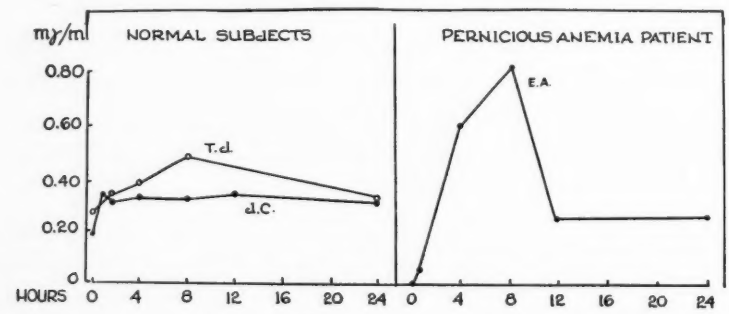

Fig. 4.—Serum vitamin B_{12} activity of normal subjects and a patient with pernicious anemia following oral administration of 1,000 μg. of the vitamin.

more prolonged than that obtained following intramucular injection of the vitamin. Maximum serum activity was observed in all subjects within four to eight hours, and was approximately equivalent to that found after intramuscular administration of 10 to 25 μg. of the vitamin. Three of the six normal subjects were allowed their usual breakfast within fifteen minutes after administration of vitamin B_{12}. The curves of serum vitamin B_{12} activity of these subjects were similar to those of the remaining three who received no food until after the four-hour sample was obtained (Fig. 5).

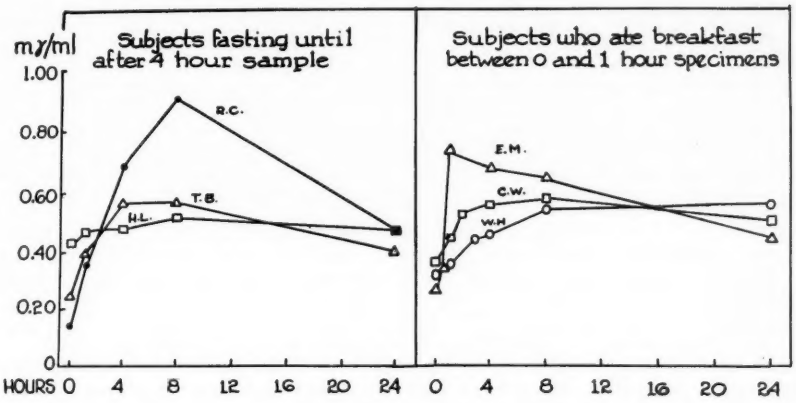

Fig. 5.—Serum vitamin B_{12} activity of normal subjects following oral administration of 3,000 μg. of the vitamin.

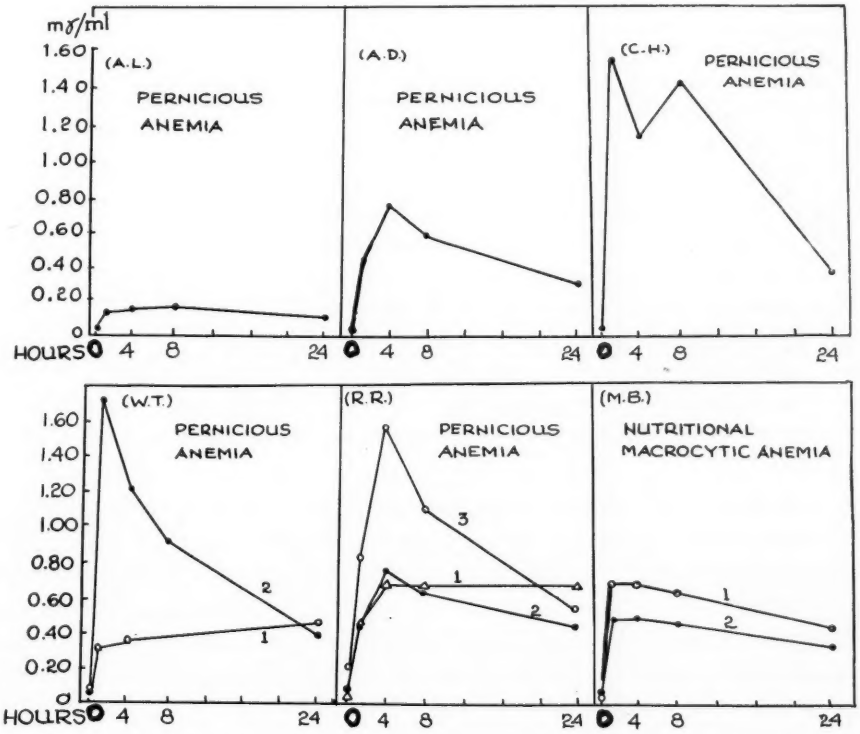

Fig. 6.—Serum vitamin B_{12} activity of patients with macrocytic anemia following oral administration of 3,000 μg. of the vitamin.

Of five patients with pernicious anemia who received 3,000 μg. of vitamin B_{12} orally, one showed a very slight increase, three moderate increases, and one a marked increase in serum vitamin B_{12} activity (Fig. 6). Hematopoietic response was satisfactory to excellent in all patients, even in patient A. L., whose serum vitamin B_{12} activity showed only minimal change (Table IV).

TABLE IV. SERUM VITAMIN B_{12} ACTIVITY AND HEMATOLOGIC RESPONSE FOLLOWING ORAL ADMINISTRATION OF 3,000 μG. OF VITAMIN B_{12} IN PATIENTS WITH PERNICIOUS ANEMIA

PATIENT	SERUM VITAMIN B_{12} ACTIVITY INITIAL MAXIMUM (MμG./ML.)		INITIAL ERYTHROCYTE COUNT (MILLIONS/ C.MM.)	RETICULOCYTE RESPONSE* (PER CENT)	ERYTHROCYTE RESPONSE* IN 2 WEEKS (PER CENT)
A. L.	0.02	0.12	1.35	100	107
A. D.	0.05	0.75	1.71	71	71
C. H.	0.02	1.54	2.19	155	82
W. T.†	0.05	0.46	1.48	90	133
	0.03	1.70	3.86	–	–
R. R.†	0.03	0.69	2.74	110	84
	0.07	0.77	2.93	172	110
	0.21	1.58	3.60	–	116
M. B.†	0.02	0.70	1.93	99	80
(NMA)	0.07	0.52	3.14	121	68

*See footnote, Table II.
†These patients received repeated doses—see text.

Two patients received 3,000 μg. of vitamin B_{12} orally on more than one occasion. Patient W. T. received four oral doses of the vitamin at intervals of four weeks, serum activity being measured after the first and fourth doses (Curves 1 and 2, Fig. 6). Serum vitamin B_{12} activity increased much more markedly after the fourth than after the first dose. The second patient (R. R.) was given 3,000 μg. of the vitamin on three occasions. The first two doses were given at an interval of one month, and similar increases in serum vitamin B_{12} activity were observed in each instance (Curves 1 and 2, Fig. 6). Two weeks following the second dose, the fasting serum vitamin B_{12} level was within normal limits, and the administration of 3,000 μg. of the vitamin produced a much more marked rise in serum B_{12} activity than was observed in the first two tests. In each instance, an excellent hematopoietic response was obtained (Table IV).

One patient with nutritional macrocytic anemia (M. B.) was given 3,000 μg. of vitamin B_{12} orally on two occasions. Serum vitamin B_{12} activity increased moderately (Fig. 6) and hematologic response was fairly good in each instance (Table IV).

Increases in urinary vitamin B_{12} activity in the twenty-four-hour period following oral administration of 3,000 μg. of the vitamin are shown in Table V. Excretion varied considerably. In 10 of 12 subjects, vitamin B_{12} activity was comparable to that found after intramuscular injection of 10 μg. of the vitamin; in the other two subjects, excretion was comparable to that found after injection of 25 to 50 μg. No definite relationship between maximum serum activity and urinary excretion of the vitamin was apparent. However, the most marked increases in vitamin B_{12} activity in the urine were observed

in the normal subject (R. C.) and the patient with pernicious anemia (W. T.) whose serum activity reached the highest level in his respective group. On the other hand, two patients with pernicious anemia (C. H. and R. R.), whose serum levels were above the level of the highest normal, showed little rise in urinary vitamin B_{12} activity. In patients R. R. and W. T., who received repeated oral doses of vitamin B_{12}, urinary activity was greater with the last dose administered than with earlier doses (Table V).

TABLE V. VITAMIN B_{12} ACTIVITY OF SERUM AND URINE FOLLOWING ADMINISTRATION OF VITAMIN B_{12}

SUBJECT	GROUP	DOSE	SERUM VITAMIN B_{12} ACTIVITY (MμG./ML.)		URINE VITAMIN B_{12} ACTIVITY (MμG./DAY)	
			INITIAL	MAXIMUM	BASAL	AFTER VITAMIN B_{12}
H.L.	Normal	3000 μg. orally	0.42	0.52	205	399
W. H.	Normal	3000 μg. orally	0.31	0.56	30	103
C. W.	Normal	3000 μg. orally	0.35	0.59	41	161
T. B.	Normal	3000 μg. orally	0.24	0.68	50	326
E. M.	Normal	3000 μg. orally	0.28	0.75	34	198
R. C.	Normal	3000 μg. orally	0.15	0.90	25	1,989
A. L.	PA	3000 μg. orally	0.02	0.12	60	43
M. B.	NMA	3000 μg. orally	0.07	0.52	27	110
	NMA	3000 μg. orally	0.02	0.70	61	331
R. R.*	PA	3000 μg. orally	0.07	0.77	24	88
C. H.	PA	3000 μg. orally	0.02	1.54	121	229
R. R.*	PA	3000 μg. orally	0.21	1.58	17	293
W. T.†	PA	3000 μg. orally	0.03	1.70	13	3,091
R. C.	Normal	10 μg. IM.	0.28	0.55	44	528
J. G.	Normal	10 μg. IM.	0.12	0.58	13	164
Til.	Normal	25 μg. IM.	0.14	0.80	46	1,213
C. J.	Normal	25 μg. IM.	0.20	1.10	16	1,658
Tis.	Normal	50 μg. IM.	0.17	1.57	29	7,266
H. T.	Normal	50 μg. IM.	0.22	2.20	51	18,315

*Urinary excretion was measured after the second and third doses of vitamin B_{12}.

†W. T. received 4 oral doses of vitamin B_{12}. Increase in B_{12} activity in the urine after the second dose was 72 mμg. in 24 hours. The increase after the fourth dose is given in the table.

DISCUSSION

Abundant evidence has accumulated which indicates that vitamin B_{12} is the antipernicious anemia factor of liver and also the "extrinsic factor" of food. However, information regarding its fate in the body and its role in metabolic processes has been difficult to obtain because of the lack of suitable methods for quantitative determination of the vitamin in tissues and body fluids. Chemical determination of vitamin B_{12}[14] and microbiological assay with a mutant form of *Escherichia coli*[15, 16, 17] are relatively insensitive methods and are not suitable for measurement of the extremely minute quantities which normally occur in body fluids. Chesterman[7] employed the *E. coli* technique for the measurement of vitamin B_{12} activity in the urine following oral and parenteral administration of the vitamin, but was unable to detect activity in the urine of normal subjects or of patients with pernicious anemia prior to administration of the vitamin. Ross[18] developed a sensitive microbiological

assay technique using *Euglena gracilis,* by means of which vitamin B_{12} may be quantitatively measured in serum and urine. He reported that the vitamin was present in the serum in both free and bound forms.

In the experience of most investigators, microbiological assay techniques which employ *Lactobacillus leichmannii* as the test organism have been successful in detecting vitamin B_{12} activity in urine only after administration of parenteral doses of the vitamin;[19, 20, 21, 22, 23] in serum, only gross changes have been detected after intravenous administration of 1,000 μg. of vitamin B_{12}.[5] Girdwood, however, found vitamin B_{12} activity in the urine of normal subjects and of patients with pernicious anemia.[24] He reported no significant difference in the urinary excretion of the vitamin between the two groups. Register and Sarett[10] also demonstrated vitamin B_{12} activity in the urine of normal subjects, and investigated the effects of various diets upon the urinary excretion of the vitamin. Girdwood[25] recently reported that, in his hands, assay with *L. leichmannii* is not capable of showing differences between serum vitamin B_{12} levels of normal subjects and patients with pernicious anemia.*

It is apparent, from results reported previously from our laboratory[26, 27] and those found in the present study, that a significant difference between serum vitamin B_{12} activity of normal subjects and patients with megaloblastic anemia can be detected by the *L. leichmannii* assay technique of Rosenthal and Sarett.[9] Furthermore, changes in serum vitamin B_{12} activity can be demonstrated following oral as well as parenteral administration of the vitamin. The difference between our findings and those reported by other investigators, who employed the same test organism, are probably due to important differences in assay technique. Our results are in general agreement with those obtained in similar studies by Mollin and Ross[28, 29] with the use of *Euglena gracilis.*

Values for urinary vitamin B_{12} activity of normal subjects obtained by our technique are lower than those reported by Mollin and Ross,[28] or by Girdwood.[24] Mollin and Ross[28] found a considerable difference in urinary vitamin B_{12} activity between normal subjects and patients with pernicious anemia. This was not observed in our studies nor in those of Girdwood.[24, 25]

In our experiments, total serum vitamin B_{12} activity increased promptly following intramuscular administration of the vitamin in both normal subjects and patients with pernicious anemia. The rise was essentially the same in the two groups. The extent and duration of alterations in serum vitamin B_{12} activity are similar to those found in pernicious anemia patients by Mollin and Ross.[29] Increases in urinary vitamin B_{12} activity following intramuscular administration of the vitamin are comparable to those found by other investigators.[5, 7, 23, 24, 25, 29, 30] Although the two patients with pernicious anemia who received 25 μg. of the vitamin exhibited somewhat less urinary vitamin B_{12} activity following this dose than did two normal subjects, the small number of observations permits no definite conclusion.

*In a letter received subsequent to the preparation of this report, Dr. Girdwood states that he has obtained, by the method of Rosenthal and Sarett, values for vitamin B_{12} activity of the serum of normal subjects which are of the same order of magnitude as those reported here. He feels that the higher values which he reported in the paper referred to above were due to substances other than vitamin B_{12} which were capable of sustaining growth of the microorganisms under the conditions of his technique.

It might reasonably be postulated, on the basis of present concepts of the intrinsic factor and its role in the absorption of vitamin B_{12}, that patients with pernicious anemia would absorb the orally administered vitamin less readily than normal subjects or not at all. That some vitamin B_{12} can be absorbed in the presumed absence of intrinsic factor is indicated by the hematologic response which follows oral administration of large amounts of the vitamin, 3,000 μg. or more, in patients with pernicious anemia.[2, 4, 6] In a summary of clinical observations, Ungley suggested that hematopoietic responses following oral doses of 3,000 μg. indicated absorption of 20 to 40 μg. or more of the vitamin. In the experience of the authors[31] and of Conley and associates,[6] oral vitamin B_{12} alone, in large amounts, can be used not only to initiate but also to maintain remission in patients with pernicious anemia.

The fate of vitamin B_{12} administered orally to normal subjects has been difficult to determine. Chesterman and associates[7] found that normal subjects excreted only 0.03 to 0.20 μg. of vitamin B_{12} in the urine after oral doses of 1,000 to 3,000 μg. They noted that this excretion was much lower than might be anticipated from the hematologic response of patients with pernicious anemia following oral doses of 3,000 μg., which suggested that as much as 100 μg. had been absorbed. If 100 μg. is given parenterally, 30 per cent or more is excreted in the urine (Fig. 1).[5, 7, 25] Chow,[19] Conley[5] and their associates failed to demonstrate any vitamin B_{12} activity in the urine of either normal subjects or patients with pernicious anemia following oral administration of 500 to 10,000 μg. of the vitamin.

Our data indicate that vitamin B_{12} is absorbed by both normal subjects and by patients with pernicious anemia following its oral administration in large doses. In general, patients with pernicious anemia have shown increases in serum vitamin B_{12} activity as great as, or greater than, those observed in normal subjects given comparable doses. Absorption of vitamin B_{12} following oral administration of large doses may occur by a different mechanism from that operative in absorption from the usual dietary sources.

The increases in serum vitamin B_{12} activity in patients with pernicious anemia following oral administration of 3,000 μg. are roughly comparable to those which follow parenteral injection of 10 to 50 μg. of the vitamin in normal subjects. Hematologic responses suggest that amounts larger than this have been absorbed. Optimal blood regeneration occurred in most subjects, whereas parenteral administration of 10 μg. rarely stimulates maximum blood regeneration, and 25 μg. often fails to do so. We have been unable to demonstrate any relationship between the maximum level of total serum vitamin B_{12} activity and degree of hematopoietic response. This is not surprising, since maximum serum activity would not be expected to reflect total vitamin B_{12} absorption. The curves showing vitamin B_{12} activity in serum after oral administration indicate that absorption continues for a considerable period of time. In some subjects, maximum blood levels were attained as late as four to eight hours after ingestion of the vitamin. The high levels persisted for longer periods following oral than following parenteral administration. It

seems likely also that the vitamin was being removed from the blood stream by the tissues during this period. In patients with pernicious anemia, urinary excretion following a single oral dose of 3,000 μg. of vitamin B_{12} was small in the few instances in which it was determined. Insufficient data are available for comparison with findings in normal subjects. However, somewhat less urinary vitamin B_{12} activity is found after oral administration than after intramuscular injection of a quantity sufficient to produce the same level of total vitamin B_{12} activity in serum. It is apparent that no strict relationship exists between the maximum total vitamin B_{12} activity of the serum and the increase in urinary vitamin B_{12} activity which follows oral administration of the vitamin. Mollin and Ross[29] have found that urinary excretion of the vitamin after intramuscular injection is greatest when relatively large amounts of the vitamin are present in the serum in an uncombined form. It seems possible that after oral administration, the majority of the vitamin present in the serum is in the combined form, and that only if the rate of absorption of the vitamin exceeds the rate of binding in the serum, or the serum binding factor becomes saturated, does the free vitamin appear in the urine.

Following repeated intramuscular injection of vitamin B_{12}, it has been found that an increasing percentage of the injected dose is excreted in the urine.[7, 23, 29] Results of the present study suggest that following repeated oral administration of vitamin B_{12}, the maximum level of the vitamin reached in the serum increases, and that larger quantities are excreted in the urine. These findings may be due to saturation of body stores, as suggested by Chesterman,[7] Sokoloff,[23] and their respective associates, or to saturation of the vitamin B_{12} binding factor of the serum, as proposed by Mollin and Ross.[29]

SUMMARY

Total serum vitamin B_{12} activity was found to be significantly lower in patients with macrocytic anemia than in normal subjects. Vitamin B_{12} activity in urine was similar in the two groups.

Following intramuscular injection of 10, 25, 50, and 100 μg. of vitamin B_{12} in normal subjects, total serum vitamin B_{12} activity increased rapidly and in proportion to the size of the dose injected. Urinary excretion of the vitamin increased roughly in proportion to the amount injected but variation was wide. Findings in two patients with pernicious anemia were similar to those in normal subjects.

Oral administration of vitamin B_{12} to normal subjects was followed by little or no change in total serum B_{12} activity after 500 μg., a slight increase after 1,000 μg., and a definite rise after 3,000 μg., which was comparable to that observed after intramuscular injection of 10 to 25 μg. of the vitamin. Oral administration to patients with macrocytic anemia in relapse was followed by a slight increase in serum vitamin B_{12} activity, to within the normal range, in 2 of 3 subjects after 500 μg. and by a definite increase in one subject after 1,000 μg. When 3,000 μg. was administered, a moderate to

marked rise in serum vitamin B_{12} activity was observed in 5 of 6 patients. A hematopoietic response followed oral administration of the vitamin in each instance but was suboptimal with doses of less than 3,000 μg.

Urine vitamin B_{12} activity following oral doses of 500 or 1,000 μg. differed little from that prior to administration of the vitamin. Following doses of 3,000 μg., an increase in urine vitamin B_{12} activity equivalent to that which followed intramuscular injection of 10 μg. of the vitamin was noted in most instances. No definite relationship between maximum total serum vitamin B_{12} activity and urinary excretion of the vitamin was apparent. Maximum levels of total serum vitamin B_{12} activity and urinary excretion tended to increase with repeated oral doses of the vitamin. No correlation was observed between maximum total serum vitamin B_{12} activity and hematopoietic response.

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LABORATORY METHODS

A SEMIAUTOMATIC RECORDING DENSITOMETER FOR USE AFTER PAPER-STRIP ELECTROPHORESIS

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IN THE course of a clinical investigation undertaken by one of us (J. D. R.) it became necessary to study the serum proteins. Paper-strip electrophoresis was suggested as a method of estimation, using a technique already described.[1] With this method it has been shown that the intensity of protein staining is proportional to concentration, when transmitted light is used.[2] This, however, necessitates rendering the paper translucent with methyl salicylate. For clinical purposes it would be preferable, if possible, to use reflected light from dry stained paper strips. This procedure would be much more rapid and simple. It was decided, therefore, to construct a scanning device which made use of either transmitted or reflected light.

The principle of reflection scanning utilized has already been demonstrated.[3] At first a straight line relationship between protein concentration and reflection density was obtained over only a limited range. The instrument has been further modified and now covers much greater ranges of protein concentration with an accuracy comparable to that obtained by transmission. It also lends itself to completely automatic scanning and simultaneous area integration.

The basic principle of the scanning device is a light bridge (Fig. 1). Two beams of light from a single source are used. One of the beams (*A*) is reflected from mirror (*B*) toward the paper strip. In the transmission model it passes through the paper and in the reflection model it is reflected from the paper to a photoelectric cell (*H*). The other beam (*C*) is reflected from a mirror (*D*) through a photo-wedge (*E*) onto the same photoelectric cell, directly in the transmission model, and by means of another mirror in the reflection model. Each of the two beams, before reaching either the paper strip or the photo-wedge, passes through one of two slits at *G*. These slits are alternately covered by an oscillating shutter (*F*), so that the light of only one beam at a time reaches the photoelectric cell. Mechanically coupled to the shutter is a switch (*I*) which is connected to the output of a resistance capacity amplifier having the photo-cell as its input. The switch has one moving and two stationary contacts, which are connected to large condensers. The switch and shutter are so arranged that the moving contact touches the stationary contacts for a shorter time than the light path associated with that contact

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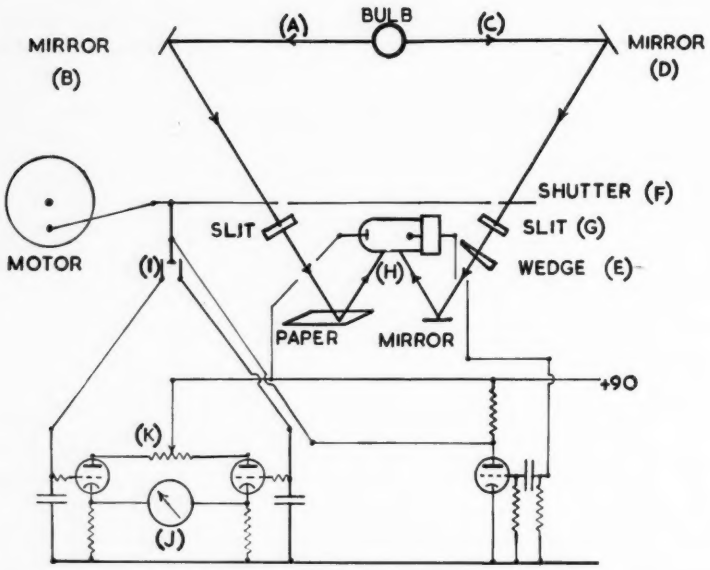

Fig. 1.—Diagrammatic representation of the basic principle of the scanning device.

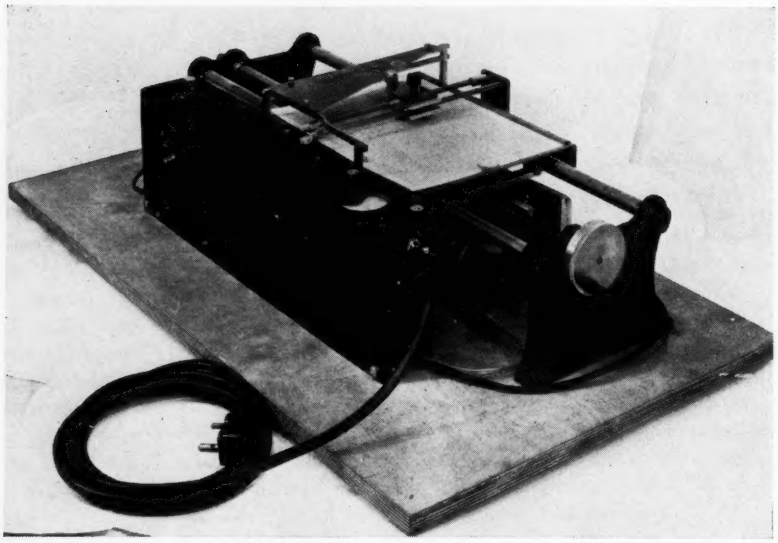

Fig. 2.—The instrument in its present form.

remains open. The voltage developed in the condensers is proportional to the intensity of the light falling on the photo-cell. These condenser voltages are compared by means of a double cathode follower with a milliammeter across the cathodes.

The scanning device itself is clearly illustrated in Fig. 2. It has a movable platform which carries on its upper surface a sheet of graph paper. Mounted above the platform is a penholder which moves along two fixed metal guide rods at right angles to the long axis of the platform. Attached to the fore edge of the platform is a frame to hold the filter-paper strip.

The photo-wedge is carried in a frame and is moved in its long axis by a simple mechanism. This involves a triangular metal wedge which is spring loaded against the penholder, so that the movement of the pen across the graph paper on the main platform causes this triangle to move at right angles to the pen movement. By a simple linkage the movement of the triangle is transmitted to the photo-wedge carrier which can thus be moved to and fro in its long axis, by moving the pen across the paper (Fig. 3).

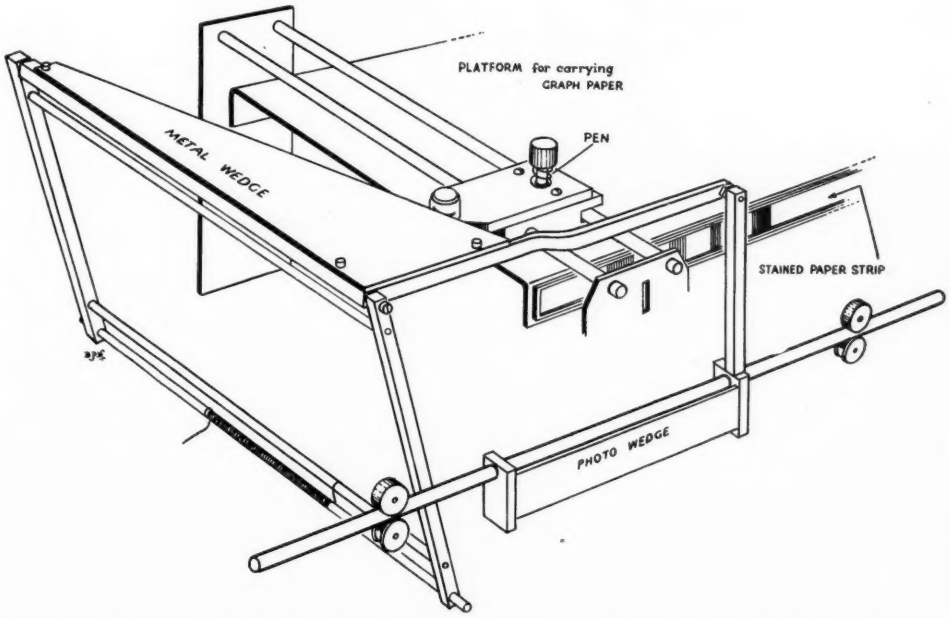

Fig. 3.—Diagram illustrating method of producing movement of photo-wedge through the agency of the pen.

The electronic circuit, the lamp, slits, and shutter, etc., are mounted in the rectangular box shown in Fig. 2. The milliammeter is mounted on the upper surface of this box. The illumination is obtained from an ordinary cinematograph exciter lamp slightly overrun. The photo-cell has been of a blue-sensitive variety, no filter has been used for the work described in this paper but, however, provision has been made for varying the photo-cell if necessary in other determinations, as well as incorporating a filter.

In operation, the filter-paper strip is placed in its carrier and a piece of centimeter graph paper is clamped onto the platform. The current is switched on and after a few minutes the circuit is balanced by means of the potentiometer (K). The light is then switched on and the meter needle takes up a position dependent on the balance of the light bridge.

The pen is moved across the paper, thereby producing the necessary movement of the photo-wedge in order to achieve balance. This is shown by a zero reading on the

milliammeter. At this point the spring-loaded pen is momentarily pressed on to the graph paper to produce an ink dot. The platform and strip are then moved simultaneously in their long axes by the lead screw which is clearly shown in Fig. 2. One revolution of the handle advances the platform 2 mm. The pen is again moved and when balance has been achieved as is shown by the milliammeter reading zero, the pen is pressed down momentarily, making a second ink dot, and the paper released. The paper is now advanced another 2 mm., and the balancing procedure repeated. In this way one eventually obtains a typical electrophoresis curve as shown in Fig. 4. The base line is previously determined from a proportion of the paper not containing any protein bands. Not infrequently small troughs are produced as shown in Fig. 4. It must be remembered that the base line is a horizontal line just above these troughs. In case of any doubt it is preferable to use the horizontal on the cathode side of the gamma globulin peak.

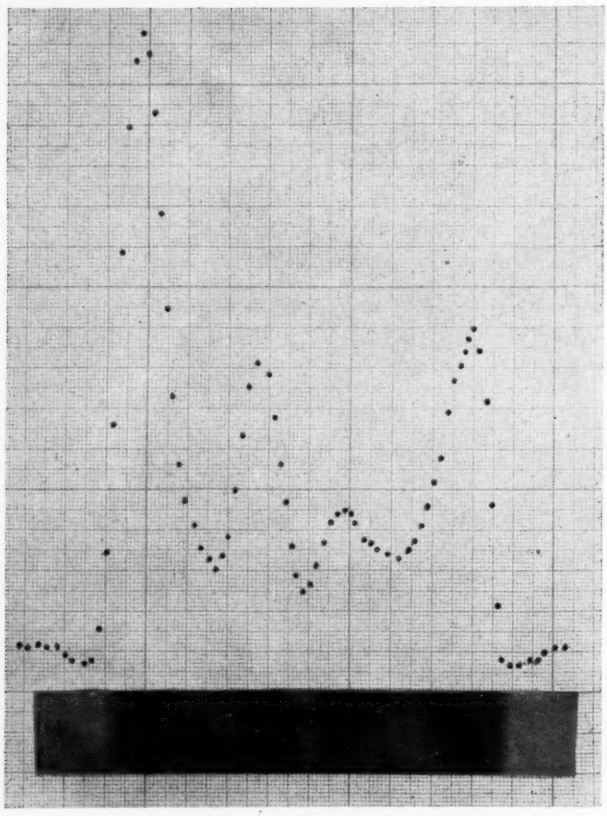

Fig. 4.—Paper-strip electrophoresis curve of serum obtained from a child with acute rheumatoid arthritis. The strip is shown below and the results of scanning immediately above it.

When it was found that the curves obtained with individual strips could be repeated we felt we were in a position to test the accuracy of the instrument in relation to actual protein concentration in the various bands. In order to do this a filter-paper reflection step wedge was constructed in the following manner.[2] A series of filter-paper strips 2 by 10 cm. were prepared from Whatman No. 100 fat-free filter paper. These strips were saturated with different dilutions of serum (0, 2, 4, 6, 8, 10, 20, 30, 40, and 50 per cent) in 0.9 per cent NaCl. This was done by suspending each strip with a drawing pin from the lower surface of the bung closing a glass boiling tube which was held

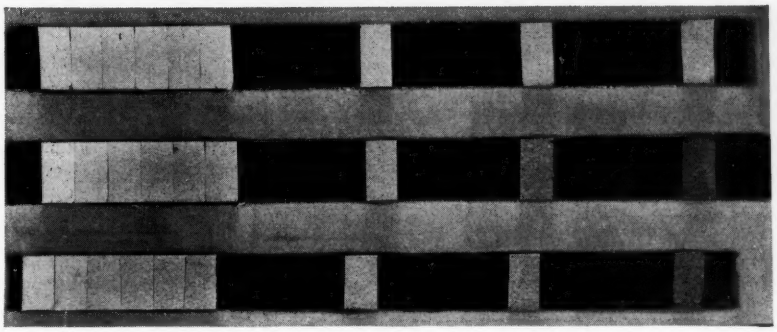

Fig. 5.—Three step wedges prepared from different grades of filter paper: the upper is from Whatman 41, and the lower two from Whatman 100 fat free.

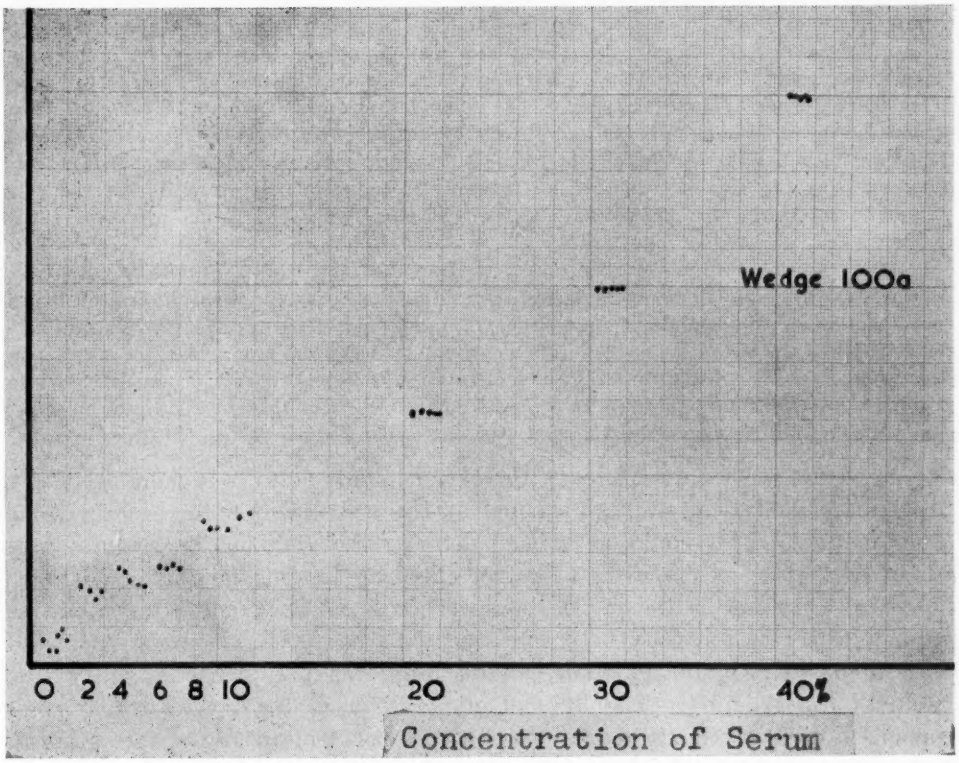

Fig. 6.—Graph of pen deflections by final model. The linear relationship between protein concentration and pen deflection obtained by scanning one of the step wedges (Whatman No. 100) with the final model of the instrument.

vertically. The lower end of the strip dipped below the surface of a small quantity of serum solution of the appropriate concentration in the bottom of the tube. The strips became saturated with the solution by means of capillary ascent. The tubes were left in the refrigerator overnight so that a state of equilibrium would be reached. The next morning the bungs were carefully removed with the filter-paper strips, which were then dried in a horizontal position in a hot air oven at 105° C.

The middle 2 cm. of each strip was cut out and treated according to our usual staining technique.[1] The strips were completely immersed in a boiling tube containing a saturated solution of naphthalene black 12B 200 (I. C. I.) in wood naphtha (Boots) containing 10 per cent glacial acetic acid (v/v). The tube was slowly rocked in a mechanical rocker for 10 minutes. The dye solution was then poured off and rapidly replaced by a mixture of 10 per cent glacial acetic acid in wood naphtha. After rapid rinsing, this liquid was also poured away. A further amount of the same mixture was poured into the tube which was closed with a rubber bung and again rocked mechanically for 10 minutes. The blue liquid was again poured off and replaced by more glacial acetic acid-wood naphtha mixture and rocked again for 10 minutes. This procedure was repeated until there had been four 10 minute periods of rocking. At this stage the liquid was poured away and replaced by wood naphtha alone and again rocked for 10 minutes. The wood naphtha was changed and the rocking repeated twice more at 10 minute intervals. The strips were now dried by suspending them before an electric fan.

Each of the resulting pieces was then cut across its middle, giving two paper rectangles 2 by 1 cm. One of these was used in the construction of a reflection step wedge. The other was rendered translucent with methyl salicylate, and used for a check by transmitted light. The reflection step wedge portions were mounted on a cardboard base in ascending order of concentration. The rectangles representing 0 to 10 per cent were placed side by side and the 10, 20, 30, 40, and 50 per cent rectangles 5 cm. apart (Fig. 5). Since a straight line relationship between extinction coefficient and concentration was obtained with the portions checked by transmitted light, it was inferred that the procedure of saturating the papers with different concentrations of serum had been satisfactory.

The reflection step wedge was then placed in the scanning device and a graph was drawn (Fig. 6). It will be seen that a straight line relationship was obtained between concentration of serum and pen deflection. This was repeated with four other similarly constructed filter-paper step wedges. The densitometer therefore recorded a linear relationship between concentration of serum and distance of the pen above the base line. Since a protein stain was used this was interpreted as meaning that there was a linear relationship between protein concentration and pen movement across the graph paper.

Now that we were satisfied with regard to this point it became necessary to compare analytical results obtained by the instrument with those by some other method. This could have been done by carrying out analyses of sera which had also been analyzed by the classical Tiselius method. There is, however, a strong criticism of this procedure. The Tiselius technique as applied to serum not only determines proteins but also the lipids associated with them. Often, for example, unduly high readings are obtained in the beta-globulin fraction. The dye technique as used for the paper strips is concerned with protein alone and is in no way influenced by the formation of lipoprotein complexes. This has been amply demonstrated by running duplicate strips and staining one before and one after ether extraction.[2] This was also confirmed by carrying out paper-strip electrophoresis of serum before and after ether extraction. The technique for staining these strips is identical with that already described in this communication.

In order to check the accuracy of the instrument, it was decided, therefore, to use mixtures of almost pure serum proteins, each constituent of which was known to be fat free and had been analyzed by the classical Tiselius method. The results with four such mixtures after paper-strip electrophoresis and their comparisons with the calculated content are shown in Table I.

TABLE I. COMPARISON OF PAPER-STRIP ANALYSES OF FAT-FREE PROTEIN MIXTURES WITH ACTUAL PERCENTAGES CALCULATED FROM TISELIUS ANALYSIS OF EACH PROTEIN

	ALBUMIN %	α-GLOBULIN %	β-GLOBULIN %	γ-GLOBULIN %
Actual	92	3	5	
Found	92	3	5	
Actual	48	2	5	45
Found	49	3	8	40
Actual	63	2	5	30
Found	59	5	9	27
Actual	5		4	91
Found	8		4	88

The instrument is rapid in action, and it is possible to scan ten paper strips in an hour. It has been in constant use for several months and has proved itself reliable in every respect. Its great advantage lies in the fact that the strips after electrophoresis and staining do not need to be rendered translucent. The graph is drawn semi-automatically, thus avoiding the tedium of plotting many readings by hand and greatly speeding up the work of analysis. We have satisfactorily rendered a model of this type completely self-recording.

Should transmission scanning be required, a simple modification of the instrument allows the insertion of a small trough which carries the filter-paper strip and the liquid in which it is immersed to render it translucent.

Although the instrument is perfectly satisfactory for serum proteins stained by the method we have described, it is probably necessary to use a different photo-cell and a light filter for other techniques. It is advisable in such a case to prepare a filter-paper step wedge as we have described but using the desired technique of staining. If a straight line relationship is obtained then the simple metal triangle we use is satisfactory. The areas under the peaks will be proportional to concentration. On the other hand, if a curvilinear relationship is obtained it is necessary to correct this. This is done by shaping the leading edge of the metal wedge into the shape of the curve obtained by using the desired staining technique.

The instrument can of course be used as a densitometer for purposes other than paper-strip electrophoresis.

SUMMARY

An instrument is described for the purpose of scanning paper strips after electrophoresis. Using reflected light, it has been completely satisfactory when put to various tests of accuracy. It is robust and easy to use.

Acknowledgment.—We are grateful to Professor Pask for the facilities of his laboratory, and much valuable advice. Mr. T. Bryson-Smith constructed the present instrument and helped in its design. Mr. C. J. Duncan and Mr. D. P. Hammersley were responsible for the photographs and drawings, respectively. We also wish to thank Mr. J. E. Rogers for technical assistance.

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AN IMPROVED TECHNIQUE FOR UROPEPSIN ASSAY

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PREVIOUS investigators[1, 2] using the hemoglobin-tyrosine technique for uropepsin assay apparently were not concerned about the serious reduction in the sensitivity of the assay which results when urines having a high content of chromogenic material are encountered. The presence of such chromogenic substances, mainly urinary phenolic compounds, has proved to be a source of serious error in our material, so serious as to make the evaluation of uropeptic activity quite impossible in a significant number of cases. This "interference" with the uropepsin estimation is not to be wondered at when one realizes that a popular technique for determination of urinary phenols[3] employs substantially the same reagents and conditions as are used in finding the value of the blank in the uropepsin estimation. In all cases having an appreciable concentration of urinary phenolic compounds, the density of color produced in the blank and in the test by the union of these compounds with phenol reagent is greater than the color produced in the test by the union of the products of proteolysis and phenol reagent. Despite retention of proportionality between chromogenic material and depth of color produced, that portion of the total color due to uropeptic activity is "swamped" by that given by the urinary phenolic compounds. Under these conditions measurement of the chromogen produced by uropeptic activity is undertaken in an optical density range which is relatively insensitive (Table I). In our experience, despite trials with different sensitive photometric equipment and with variations in reagent concentrations, the optical density differentials indicating uropeptic activity appear to be much below true values. Any attempt at diminishing the concentration of phenolic compounds by dilution of the urine sample results in concomitant diminution of uropeptic activity often almost to the vanishing point and fails to improve the sensitivity of the method.

TABLE I. EFFECT OF DIALYSIS ON CHROMOGENIC VALUE OF 5 UNSELECTED URINE SAMPLES

	AFTER DIALYSIS		NO DIALYSIS	
URINE	BLANK	TEST	BLANK	TEST
1	0.143	0.387	0.796	1.026
2	0.131	0.347	0.745	0.854
3	0.119	0.292	0.620	0.745
4	0.125	0.215	0.420	0.444
5	0.131	0.319	0.252	0.398

After trials of various methods for reducing the concentration of phenolic compounds in the urines, dialysis through a viscose membrane has been found to rid the urine of practically all the interference from phenolic compounds. At the same time it has been found that uropeptic activity is retained without diminution (Tables II and III).

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TABLE II. ACTION OF DIALYSIS ON CONCENTRATION OF TOTAL CHROMOGENIC MATERIAL, EXPRESSED AS OPTICAL DENSITY, IN TWO URINE SAMPLES

	URINE A			URINE B		
	BLANK	TEST	T-B	BLANK	TEST	T-B
No dialysis of urine	0.886	1.046	0.160	0.328	0.432	0.104
Dialysis in 50 ml. water	0.220	0.398	0.178	0.137	0.252	0.115
Water of this dialysis	0.237	0.237	0	0.131	0.131	0
Dialysis in 500 ml. water	0.092	0.292	0.200	0.092	0.229	0.137
Water of this dialysis	0.086	0.086	0	0.081	0.081	0
Dialysis in 1000 ml. water	0.081	0.284	0.203	0.086	0.226	0.140
Water of this dialysis	0.081	0.081	0	0.076	0.074	0
Water and substrate only	0.066	0.066	0	0.066	0.066	0

TABLE III. UROPEPTIC ACTIVITY OF 10 URINE SAMPLES WITH AND WITHOUT DIALYSIS; MILK TECHNIQUE

URINE	DIALYZED			AVERAGE	NOT DIALYZED			AVERAGE
1	200	195	180	192	205	203	181	196
2	320	314	310	315	310	315	303	309
3	325	331	308	321	328	323	311	321
4	94	82	80	85	90	81	98	90
5	113	106	103	107	104	111	107	107
6	168	159	143	157	163	167	141	157
7	113	118	104	112	120	108	114	114
8	205	211	201	206	203	208	216	209
9	88	84	77	83	80	87	79	82
10	83	74	81	79	79	78	86	81

Method for Dialysis and Assay.—A 20 ml. aliquot of urine is placed in dialyzing cylinder made of "sausage casing" viscose cellophane tubing, the cylinder is suspended by a thread and glass rod and immersed for twenty-four hours in a beaker containing 500 ml. of distilled water and placed in a refrigerator at 4° C. Following dialysis the urine is adjusted to pH 1.5 with a pH meter, using 3N hydrochloric acid and made up to a total volume of 25 ml. with distilled water. The same adjustment of pH and volume, but without dialysis, is carried out on another 20 ml. aliquot of the same original urine sample. In this way two aliquots of adjusted urine are obtained, one of them dialyzed, the other preserved in the refrigerator without dialysis. The method employed for assay of enzyme follows in general the techniques used by Bucher[1] and by Mirsky and associates,[2] both of which were found to be entirely satisfactory for measurement of the enzyme, following prior removal of nonenzyme chromogenic material.

DISCUSSION

Columns 1 and 3 of Table I illustrate the effect of dialysis on the concentration of chromogenic (phenolic) compounds in five unselected urine samples. The figures given are optical density readings. The samples were treated and read as detailed in "Method for Dialysis and Assay," where applicable.

Study of Table II reveals the following: (1) Dialysis removes most of the interfering chromogenic material. (2) As increasing quantities of water are used in the dialyzing container more interfering chromogenic material is removed from the urine. (3) A satisfactory quantity is removed by using 500 ml. of dialysate for twenty-four hours. (4) The substrate contains chromogenic material which is too low in concentration to interfere with uropepsin assay.

(5) The substrate itself contains no proteolytic factor. (6) A small quantity of chromogenic material remains in the urine sample after dialysis but this is too low in concentration to interfere with the assay.

In order to satisfy ourselves that the increase in uropepsin values demonstrated following dialysis was not due to increased proteolysis introduced by altered conditions of handling the urine specimen, such as perhaps the removal of an enzyme inhibitor, a series of ten urine samples was examined both with and without dialysis, employing the milk substrate technique.[4] In Table III, columns 4 and 8 are the averages of the triplicate determinations shown in the three preceding columns. The figures, which represent the number of seconds required for the enzyme to produce precipitation of the milk protein, give a quantitative evaluation of uropeptic activity and indicate that this activity is unaltered by dialysis.

Table IV shows the effect of dialysis in increasing the amount of uropepsin demonstrated in 46 male patients, 18 to 40 years of age, with minor surgical conditions or minor upper respiratory tract disease. The numbers, arranged in diminishing order of uropeptic activity, represent units of uropepsin found in first morning urine samples, expressed as micrograms of tyrosine per milliliter of undiluted urine, i.e., tyrosine-like material released by the amount of enzyme contained in 1 ml. of original urine sample. In several cases the amount of uropepsin demonstrated following the removal of interfering chromogens increased by several hundred per cent over the amount found without dialysis. The six cases in this series in which no uropepsin was found in the undialyzed urine all had moderate or high concentrations of interfering chromogens combined with low uropepsin content with resultant "swamping," which masked completely the uropepsin subsequently demonstrated after dialysis.

TABLE IV. COMPARISON OF UROPEPSIN VALUES WITH AND WITHOUT DIALYSIS IN 46 MALE PATIENTS; HEMOGLOBIN TECHNIQUE

CASE	AFTER DIALYSIS	NO DIALYSIS	CASE	AFTER DIALYSIS	NO DIALYSIS
1	560	460	24	170	168
2	544	432	25	164	56
3	540	440	26	150	40
4	504	242	27	150	76
5	484	408	28	150	76
6	460	326	29	140	0
7	460	225	30	132	0
8	416	380	31	122	86
9	405	385	32	122	74
10	398	376	33	112	30
11	360	185	34	110	84
12	358	206	35	110	104
13	330	230	36	102	94
14	320	282	37	90	56
15	302	230	38	74	28
16	285	210	39	74	72
17	282	48	40	66	66
18	270	204	41	64	64
19	244	240	42	56	0
20	234	104	43	56	0
21	226	226	44	48	0
22	226	188	45	46	0
23	196	150	46	46	46

SUMMARY

Hemoglobin substrate techniques used heretofore exhibit serious errors of assay in those cases having normal and high urinary phenol content. A simple dialysis technique is presented for ridding the urine of interfering chromogenic material without loss of enzyme. The hemoglobin substrate method used with prior removal of urinary phenols by dialysis provides, we believe, the most satisfactory precise and reproducible method for uropepsin assay yet devised.

Acknowledgment.—We are indebted to Dr. D. Hugh Starkey of Montreal for suggesting the use of dialysis for the elimination of phenolic compounds from the urine.

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Erratum

In the October, 1953, issue of the Journal, in the article entitled "Ethylenediaminetetraacetic Acid in the Mobilization and Removal of Iron in a Case of Hemochromatosis," by Henry Wishinsky, Ph.D., Tobias Weinberg, M.D., Eleanor M. Prévost, A.B., Bernard Burgin, M.D., and Max J. Miller, M.D., in Fig. 1, on page 552, the scale should read from 1.0 to 5.0 instead of 10 to 50 mgms/24 hours.

Reference 2, on page 554, should read Bull. New York Acad. Med. instead of Bulletin of the New York Academy of Sciences.